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Primary Cultivation and Continuous Propagation *In Vitro* of Tissues From Small Biopsy Specimens[1,2]

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SUMMARY

A method is described for cultivation of small biopsy specimens *in vitro* with high frequency of success. A relatively safe method for transferring and propagating cells is also described. These procedures enable investigators to establish cell lines from limited quantities of initial material. In securing cellular material from human hereditary diseases for cytological and/or biochemical investigations, these techniques may be helpful.—J. Nat. Cancer Inst. 25: 221–235, 1960.

IN STUDIES of the chromosome constitution of mammalian cells there are distinct advantages to using cells *in vitro* instead of tissues *in situ*. A difficulty in studying chromosomes *in situ* is that we are limited to only a few types of tissues that show constant mitotic activity, such as embryonic tissues, bone marrow, and lymphatic organs. In tissue cultures, besides the advantage of securing a higher rate of mitosis, monolayers of cells can be planted so that the hypotonic-solution treatment can be most effective and fixation can be at its best. The correct human idiogram, for instance, could be constructed only after modern cytological techniques had been perfected on short-term and long-term cells in culture (*1–4*).

Recent advances in human cytology have revolutionized some chapters of medical genetics. The discoveries of an XXY condition in the Klinefelter syndrome (*5*, *6*), of an XO condition in gonadal dysgenesis (*7*, *8*), and of the trisomic nature in mongolism (*9*, *10*) stimulated keen interest in both biological and medical circles. Our laboratory has been repeatedly asked by various hospitals and laboratories in this country for assistance in their cytological and tissue-culture work. Even though bone marrow has been the conventional material for such a study, we prefer such other tissues as the skin because of the cytological clarity of the fibroblasts. Consequently, cultivation *in vitro* is necessary for such studies.

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During recent years voluminous information has been obtained on the chromosomes of transplantable tumors and of cell strains. Most investigators feel, however, that these materials have a long history of evolution; consequently, their present cytological pictures may not be representative of their original cell types. Therefore there has been a growing interest in studying the chromosome constitution of primary tumors, especially those of man. Only a few reports are available due to the difficulty in obtaining good preparations from tumors *in situ* (*11–13*). Many workers have resorted to the second choice of determining the deoxyribonucleic acid content (*14–16*). A solution to this problem would be to obtain a greater percentage of success in cultivating primary tumors *in vitro*. Our recent experience with normal human and rat tissues in culture has assured us that such cells rarely deviate from their original cytological constitution for any considerable period. It is thus surmised that information collected from primary cultures of tumors should also reflect the situation *in situ*.

There are many methods available for growing biopsy or autopsy specimens. The classical method is to use a plasma clot to provide both nutrient and a lodging substance for the tissue fragments. This method usually gives a relatively high yield, but the clot is always a nuisance in photography, cytological preparation, and subculture of the tissue fragments. Most investigators try to avoid the clot and simply use floating tissue fragments in culture vessels. Although the cells may remain viable in the fragments for a considerable period, they usually fail to emigrate in sufficient quantity to facilitate any type of study. Trypsinization of fragments before cultivation works well with some tissues such as monkey kidney, but with tissues rich in collagenous fibers it generally fails to dissociate the cells. Besides, overtrypsinization plus agitation such as the use of a magnetic stirrer may cause so much cellular damage that there is an extremely poor yield *in vitro*. Success has been claimed by Tjio and Puck (*17*), however, who used trypsin in combination with plating techniques. A new method of using collagenase has been devised by Hinz and Syverton (*18*) and according to the report the resulting growth was excellent. These methods are not simple and collagenase is rather expensive.

We present simple but effective methods for cultivating small tissue fragments. Actually, all the techniques described herein have been devised by earlier investigators; it seems to be the proper combination of media, culture handling, etc., that makes our composite method effective. To enable small laboratories to grow tissues *in vitro* without purchasing expensive supplies and equipment, we shall suggest the simplest and most inexpensive items possible and, for convenience, include the names of commercial corporations from which we purchase our supplies. This does not mean that these are the only good products. Undoubtedly, satisfactory substitutes can be obtained elsewhere; even in our laboratory changes are constantly made whenever we find a better product.

MATERIAL

For skin biopsies, disinfectants containing iodine or mercuric compounds should be avoided. Biopsy specimens as small as 2 to 3 mm. in diameter can be used to initiate a cell line. The tissues to be cultivated should be set up for cultures as soon as practicable. If circumstances do not permit immediate cultivation, the tissues should be refrigerated in growth medium.

Growth Medium

The advantage of using such media as 199, 1066, NCTC 109, and Eagle's is that they can be purchased. These are useful when laboratories are not equipped to prepare media from the basic ingredients. Even though we have not had occasion to use 199 or NCTC 109 they are undoubtedly competent media, especially the latter. Limited experience with 1066 has shown it to be quite acceptable for primary explants. Eagle's formula, however, does not always stimulate good growth in primary cultures, even though it is excellent for cell lines such as strain L. At least 10 percent serum by volume is necessary for all cultures except a small number of cell lines. For primary cultures, 20 percent serum is recommended. Calf serum (Colorado Serum Co., Denver, Colorado) and fetal calf serum (Microbiological Associates, Bethesda, Maryland) are both effective, especially the latter. A number of laboratories use cord serum, which is probably the best of all serums.

We found McCoy's formula 5a (*19*) satisfactory as a general medium. Established cell lines and primary cultures alike proliferate well on this medium. The formulation was designed for cell cultivation under an atmosphere containing 5 percent CO_2. When partial pressure is absent, under normal incubation temperatures of 35 to 37° C., a rapid *p*H rise to extreme alkalinity occurs. This undesirable and toxic alteration of *p*H can readily be prevented by gassing the medium with carbon dioxide and storing the gassed medium at 4° C. Difficulties with carbon dioxide tension can also be partially circumvented by applying Gwatkin and Siminovitch's (*20*) modification to the 5a medium. The modification replaces carbon dioxide and bicarbonate with oxalacetate and ferrous sulfate. This works quite well with the 5a formula on strain L. For basic ingredients of medium formula 5a, *see* Appendix 1.

The ingredients are weighed out singly, preferably in no less than 10 liter quantities to reduce weighing errors. Double- or triple-distilled water is used throughout. The amino acids are dissolved in 50 percent of the total volume of distilled water. It is necessary to use a magnetic stirrer and hot-plate combination (Temco StirPlate), with a slow stirring speed and with the temperature set between 30 and 40° C., to dissolve the amino acids. The minerals, phosphate buffers, and sodium bicarbonate are dissolved separately in equivalent quantities of the remaining distilled water. They are then added, in the order mentioned, to the amino acid solution by stirring. The vitamins, usually in a 100 ×-con-

centrated solution, and the phenol red indicator are then added, by stirring, to the mixture just prepared. The solution is gassed with pure carbon dioxide until the indicator is yellowish orange. The use of a coarse glass filter to disperse the gas in the solution is very effective. The antibiotics and serum are stirred into the solution which is then filtered through a size C Seitz filter pad. The use of vacuum in accordance with the manufacturer's suggestions will eliminate excessive frothing. The medium is immediately distributed to bottles of desired volume and refrigerated. The bottles should be stoppered or capped tightly to prevent the escape of carbon dioxide. No loss of effectiveness has been noted over a 2-week storage period.

Cultures

Primary cultures

The tissues should be cut with sharp surgical blades or minced with scissors into small fragments. As soon as this is done, the fragments are transferred into culture bottles with the aid of disposable Pasteur pipettes (Harshaw Scientific Co., Cat. #H–55699). At this stage only a little medium is used to wet the floor of the vessel. The fragments can be roughly distributed over the floor of the vessel either by the same pipette or by a platinum hook.

The 2 ounce screw-capped medicine bottles (Owens Ovals, Owens, Ill., Cat. #P–302) are very satisfactory. The bottles can be used directly from the shipping box without washing or sterilization. There has been no microbial contamination indigenous to these bottles in our laboratory. The bottles are so inexpensive (less than 4 cents each) that it is considered more economical to discard them after use than to rewash and resterilize them. Owens Ovals have 2 minor disadvantages: (1) The top side is oval-shaped so that the bottles cannot be stacked up, and (2) their optical quality is poor. The former defect can be remedied by installing more shelves in the incubator. The short-necked T-series flasks manufactured by Corning Glass Company could be used if optical quality is to be emphasized. When the quantity of material is too small, the T-15 flasks (inverted neck type manufactured by Bellco, Vineland, New Jersey) are excellent.

Cut a piece of perforated cellophane (Microbiological Associates, Bethesda, Md.) approximately the same area as the floor of the bottle. The perforated cellophane method was introduced by Evans and Earle (*21*) and has been widely used since. Actually, dialysis membrane or other sheets of cellophane can also be used. The perforation is not a necessary feature. Sterilize the cellophane sheet by boiling it in water or soaking it in 70 percent alcohol. Use 2 pairs of forceps, one to hold the sheet and the other to roll it so that it can be pushed through the neck of the bottle. We simply disinfect our hands with alcohol and roll the cellophane with our fingers. Many investigators would gasp at such a practice. Even though we would not recommend this as a safe procedure, we still think the manipulation is much easier with the fingers.

After the roll is placed in the bottle, it can be unrolled by a pair of long forceps. Press down the cellophane sheet lightly against the floor of the bottle so that the tissue fragments are directly covered by the cellophane sheet. Add 5 to 7 ml. of growth medium to each Owens Oval, cap the bottle tightly, and incubate.

The tissue fragments generally begin to show cellular emigration a few days after setting the cultures. The time required for initial growth varies according to the type of tissue, age of donor, number of fragments, and the cleanliness of cutting. Growth medium can be changed once or twice a week until the attainment of confluent cell colonies.

Subculture

When there is thick growth around the explants or when colonies merge, the culture is ready for cytological preparation or for subculturing. Most cells cannot withstand mechanical scraping. Unless it has been tested prudently with spare cultures to prove that scraping with a rubber policeman or other instruments does not damage the cells, trypsinization is the preferred procedure.

Many laboratories have their own method of trypsinization. After testing various ways and losing a number of stocks, we found the following method quite safe and effective. Prepare a stock 1 percent trypsin solution by dissolving "trypsin 1:300" (Nutritional Biochemicals, Cleveland, Ohio) in balanced salt solution. Filter, sterilize, and store 20 ml. each in 2 ounce Owens Ovals in a freezer. Before use, one bottle is thawed and the contents mixed with 80 ml. of growth medium to make a 0.2 percent solution. The *p*H of the 0.2 percent trypsin solution is adjusted to 7.2. This solution is kept under refrigeration. In strains whose cells attach to the glass firmly, either increased alkalinity of the trypsin medium or the conventional trypsin preparation, namely, 0.2 percent in Ca- and Mg-free salt solution, can be applied. Generally speaking, cultures composed mainly of spindle cells do not need such harsh treatment.

Decant the growth medium from the culture bottle and add approximately 5 ml. of the 0.2 percent trypsin medium. Incubate for approximately 30 minutes in which time most cells should be dislodged. Use a pair of forceps to lift up the cellophane sheet, wash off attached cells, and discard the cellophane. Break the cell clump with the aid of a Pasteur pipette. Cells not dislodged by trypsin can also be pushed off the glass by this procedure. Centrifuge the cell suspension, decant the supernatant, and resuspend the cells in growth medium. It should be remembered that most cells do not multiply well when seeding is light. It is probably a preferred procedure for the first subculture to combine 2 bottles of cells to stimulate fast growth. No cellophane sheet is necessary in subculturing, and the original tissue fragments can still be included in the subculture bottles. The free cells usually attach to the bottle in less than an hour.

Chromosomal Techniques

Chromosomal techniques used in our laboratory have changed only slightly from the methods described by Hsu and Klatt (*22*). A few points worth discussion are presented:

1. Different animals and types of cells respond to colchicine differently. A fair start to test the proper concentration of colchicine is 0.5 μg. per ml. medium. In L cells, 2 μg. per ml. gives excellent results, but for most human cells the concentration should be reduced since they are rather susceptible to colchicine. The duration of colchicine treatment should likewise vary according to individual cases. Our system of overnight treatment works well with L cells but has failed in many other strains. For instance, the mammary-tumor line 2940, supplied by Dr. Katherine K. Sanford, of the National Cancer Institute, was extremely susceptible to colchicine (*23*). The chromosomes were severely contracted and became unobservable after colchicine treatment at a concentration of 0.5 μg. per ml. for no longer than 6 hours. For such material, therefore, a reduced concentration and a shortened treatment period should be used. With Syrian hamster tissues, a high concentration of colchicine is necessary.
2. The derivative of colchicine, Colcemid (CIBA Pharmaceuticals, Inc., London, England), can be used to replace colchicine with excellent results.
3. In order to get a high number of mitotic figures, a culture must be in its logarithmic growth period. Microscopic inspection is helpful for ascertaining the suitable time for harvest.
4. After the desired duration of colchicine or Colcemid treatment, the medium is decanted and trypsin solution is added to dislodge the cells. This procedure not only dissociates the cells but also removes extracellular proteins and dead cells. The final preparation is much cleaner than a preparation made with mechanical scraping.
5. Fixation is performed in 12 ml. conical centrifuge tubes after pretreatment with hypotonic solution. A small amount of material, not more than 3 or 4 million cells, usually gives best fixation. Tightly packed cells, surprisingly enough, give better fixation than cell suspensions.
6. Appendix 2 is a synopsis of our present procedure for chromosome squash preparations, and figures 1 and 2 are examples of cells from mouse and human, respectively, to illustrate the quality of such preparations. It must be added that these represent cells of average quality only. We did not select the best pictures in our file for this purpose.
7. A modification of the air-dry method (*24*) gives reasonably good results with stationary types of cultures; *see* Appendix 2 for details. For laboratories desiring to study chromosome constitutions of certain cells but unable to support a competent cytologist, the air-dried specimens are especially useful because they can be safely shipped to cytologists in other laboratories.

Levan and Biesele (*26*) found that trypsinization increased the frequency of chromosomal breaks in mouse-tissue cultures. In our laboratory, Dr. Levan, as a guest investigator, found that trypsinization of the rat-embryo cultures did not seem to induce a high frequency of breakages (personal communication). Either the rat cells are more resistant to trypsinization than those of the mouse or our trypsin preparation is more gentle because we used growth medium as diluent.

By no means do we consider the procedures described here the best for cell cultures. Many available methods may provide richer growth. Comparatively, our method gives slower initial growth than others. However when other methods fail, or when the investigator does not wish to risk small quantities of biopsy material with harsh treatments, the present techniques may be safer and give higher chances of success.

There is no doubt that improvements will continue to be made, and each laboratory should indeed make its own modifications to fit its particular needs.

RESULTS

More than 60 specimens (including human bone marrow, skin, gingiva, testes, tumors; mouse kidney, bone marrow, omentum, adrenal glands, blood vessels, ovary, lung, spleen, mammary tumor; and rat embryonic tissues) have been tested. Successful initiation of growth has been obtained from a skin biopsy shipped from Baltimore, Maryland, to Houston, Texas, 3 days after operation. All of the specimens showed good initial growth. Many of these were able to propagate for a number of transfers. Attempts to grow adult mouse liver and cerebellum, however, failed to yield growth.

DISCUSSION

The disadvantage of using *in vitro* cells for chromosome studies is the possibility of heteroploid transformation which, if it happens, may give misleading results. Actually, as we now know, many of the so-called transformations or alterations of cell lines were in reality cellular contaminations. The fact that our cell lines derived from normal tissues remained predominantly classical diploid for at least 6 months indicates that it is quite safe to use the first few transfers as material for karyotypic determination, as well as for other biological and biochemical researches on normal mammalian cells. In work with cells of the Chinese hamsters *in vitro*, Yerganian and Leonard (*25*) expressed the same feeling.

The real hazard in using tissue-culture cells for karyotypic studies involves tumors because there is no safe way to completely eliminate the contamination of stroma elements, thereby confusing the results. This happened recently in our laboratory when normal rat chromosome complements appeared in a primary culture of the cytologically characteristic Novikoff hepatoma. In tumors of epithelial nature, cellular morphology can probably assist in determining the majority of the cell types in the culture, but in tumors of connective tissue origin this task would be more difficult.

ADDENDUM

The paper of D. G. Harnden on cultivation of human skin specimens appeared in the *British Journal of Experimental Pathology* (vol. 41, pp. 31 to 37, 1960) after our manuscript had been sent to the press. His method is very similar to ours except that he uses plasma clot, whereas we try to avoid it. Another recent development was made by Paul S.

Moorhead, of the Wistar Institute, Philadelphia, who obtained excellent results in cultivating peripheral blood for cytological analyses. His cytological preparations from blood cultures are indeed superb, and his method is almost foolproof. However it is not known whether blood cultures can be continuously propagated for an endless supply of cells. Skin cultures, therefore, still have their place.

APPENDIX 1

Composition of Medium 5a

Amino acids	Mg./liter
L-Tryptophan	3. 1
L-Phenylalanine	16. 5
L-Tyrosine	18. 1
L-Arginine·HCl	42. 1
L-Histidine·HCl·H_2O	20. 9
L-Lysine·HCl	36. 5
L-Cysteine	31. 5
L-Methionine	14. 9
L-Isoleucine	39. 3
L-Leucine	39. 3
L-Valine	17. 6
L-Threonine	17. 9
L-Glutamine	219. 2
L-Asparagine	45. 0
L-Glycine	7. 5
L-Serine	26. 3
L-Alanine	13. 4
L-Proline	17. 3
L-Hydroxyproline	19. 7
L-Aspartic acid	19. 9
L-Glutamic acid	22. 1

Minerals	Gm./liter
NaCl	6. 46
KCl	0. 4
$MgSO_4 \cdot 7H_2O$	0. 2
$NaH_2PO_4 \cdot H_2O$	0. 58
$NaHCO_3$	2. 20
$CaCl_2$	0. 1

Vitamins	Mg./liter
Thiamine·HCl	0. 2
Riboflavine	0. 2
Pyridoxine·HCl	0. 5
Pyridoxal·HCl	0. 5
Nicotinic acid	0. 5
Nicotinamide	0. 5
Calcium pantothenate	0. 2
Biotin	0. 2
Folic acid	0. 2
Choline chloride	5. 0
Inositol	36. 0
p-Aminobenzoic acid	1. 0
Ascorbic acid	0. 5
Glutathione	0. 5
Vitamin B_{12}	0. 00075

Antibiotics	Mg./liter
Potassium penicillin G	50. 0
Streptomycin sulfate	50. 0
Neomycin	100. 0

Miscellaneous	
D-Glucose	3.0 gm./liter
Phenol red	10.0 mg./liter

Protein	
Serum protein (usually human or calf, preferably fetal calf)	20%

APPENDIX 2

Procedure for Squash Preparation of Cells *in Vitro*

I Reagents:

1. *Colcemid solution.*—Colcemid from CIBA Pharmaceuticals, London, England. Prepare a 2 μg. per ml. stock solution by dissolving the compound in balanced salt solution. Filter, sterilize, and keep the stock solution in refrigerator.
2. *Trypsin solution.*—Trypsin 1:300, Nutritional Biochemicals, Cleveland, Ohio. Prepare a 0.25 percent trypsin solution in Hank's solution without Mg and Ca. Keep in refrigerator.
3. *Hypotonic solution.*—Prepare a 1 percent sodium citrate solution in distilled water. This solution needs no refrigeration.
4. *Fixative.*—Fifty percent glacial acetic acid in distilled water.
5. *Acetorcein.*—A 2 percent orcein solution dissolved in 50 percent acetic acid. Boiled for 30 minutes, filtered when cool.
6. *Sealing wax.*—"Deckglaskitt nach Krönig."

II Procedure:

1. Select growing cultures showing abundance of mitosis by microscopic inspection at low magnifications.
2. Add 0.03 ml. of Colcemid solution to every ml. of medium. Incubate the culture for 1½ to 4 hours.
3. Decant the Colcemid medium, add trypsin solution to cover the floor of the flask. Incubate for approximately 15 minutes to dislodge the cells. For suspension cultures this step may be omitted.
4. Pour the cell suspension into centrifuge tubes (preferably 15 mm. conical tubes). Break cell clumps, if any, with a pipette. Centrifuge for approximately 5 minutes. Decant the trypsin solution. Add approximately 10 ml. of hypotonic solution to each centrifuge tube. Resuspend the packed cells with a pipette. Leave the cells in the hypotonic solution for 5 to 10 minutes at room temperature.
5. Centrifuge the cell suspension in the original tubes. Decant hypotonic solution. Drain thoroughly by placing the mouth of the centrifuge tube against a piece of an absorbent paper. Add fixative (approximately 5–7 ml.) without disturbing the packed cells. Fix for at least 20 minutes at room temperature.
6. Remove fixative completely. Place 4 to 5 drops of acetorcein into the tube. The amount of stain added depends on the size of cell pellet. Remember it is easier to add a few more drops than take them away. Suspend the packed cells with a Pasteur pipette.
7. Squash preparations can be made immediately after the cells are suspended. Place a small drop of stained cell suspension on a clean slide, cover with a coverslip (preferably 22 × 30 mm. rectangular), absorb excess stain with a filter paper. Press thumb

on the filter paper over the coverslip. If the coverslip moves under pressure, discard the preparation. Examine immediately with a phase microscope to see whether the cells are well pressed out but not broken and whether the cells are overly crowded. Adjust the pressure until an ideal preparation is made. Use the same pressure for the remaining slides.

8. Seal the edges of the coverslip with Krönig coverslip wax.

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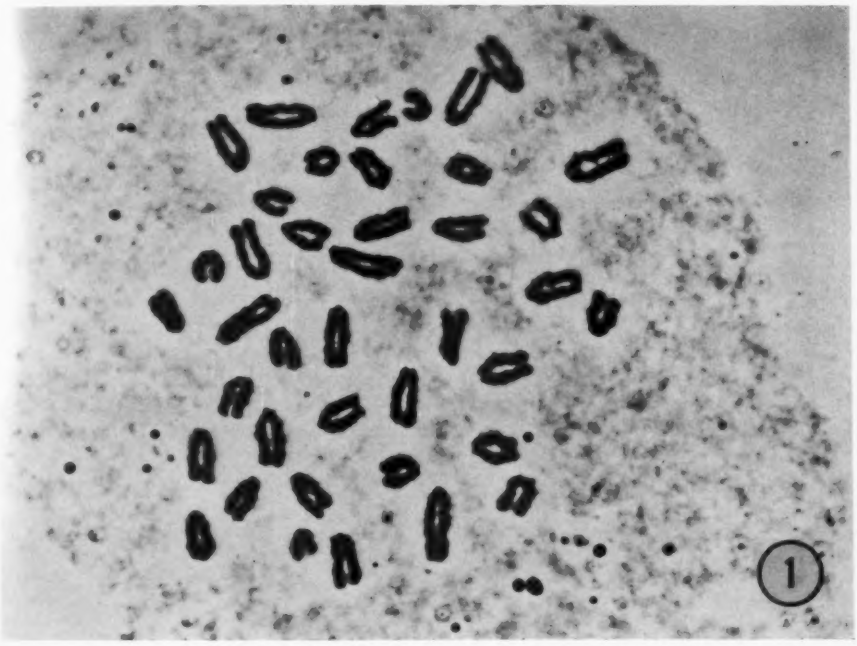
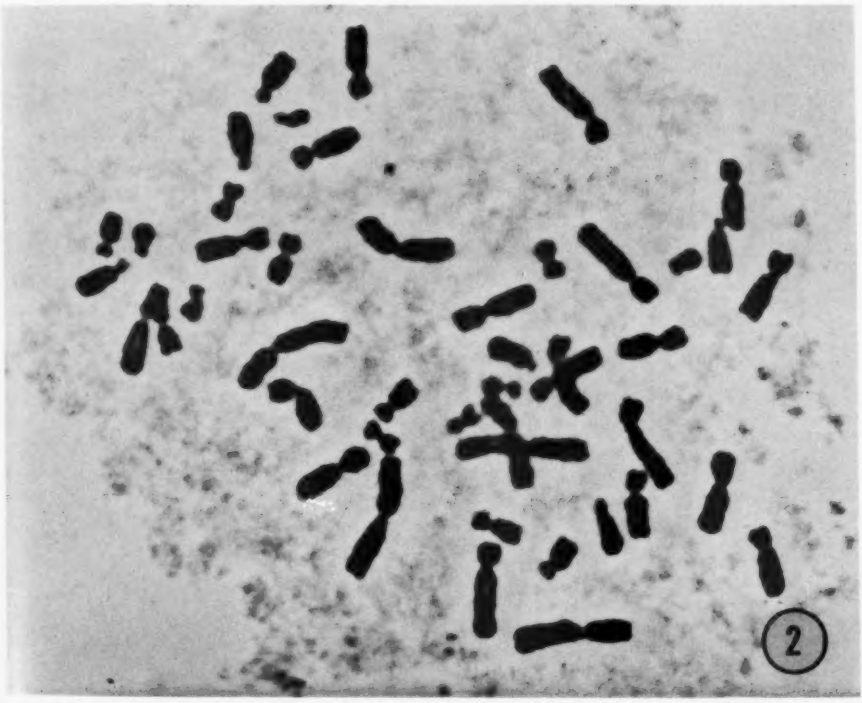
PLATE 25

FIGURE 1.—Cell from a line derived from the peritoneal wall of a female C3H mouse. Forty chromosomes.

FIGURE 2.—Cell from a line derived from a gingiva biopsy of a girl. Forty-six chromosomes.





553742—60——2

Studies on Isoantigenic Variation in Mouse Lymphomas [1, 2]

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SUMMARY

Studies on isoantigenic variation in lymphomas originating in homozygous and heterozygous F_1 hybrid mice were made by serologic, chromosome cytologic, and transplantation tests. The homozygous lymphomas grew only in their genotype of origin, while the F_1 lymphomas gave rise to progressively growing variant sublines, selectively compatible with one or the other of the parental strains. Serologic tests by the method of Gorer and O'Gorman showed that the variants had lost their sensitivity to cytotoxic antibodies directed against the *H-2* isoantigens derived from the opposite parental strain. This sensitivity could not be restored by serial passages of the variant subline in the F_1 genotype of origin or in the opposite parental strain for 12 to 17 transfer generations. Homozygous lymphomas and the variants derived from heterozygous lymphomas were slightly more sensitive to the cytotoxic effect of *H-2* isoantibodies than the F_1 lymphomas were. It was not possible to select any isoantibody-resistant cells by 4 consecutive *in vitro* treatments of an F_1 lymphoma, with antibodies directed against one of the parental strain components in the presence of complement. No chromosomal differences were found between the parental strain variants and the F_1 tumors of origin in 2 cases examined. The permanence of the changes observed suggests that the mechanism of variant formation is genetic and possibly due to mitotic crossing over. However, epigenetic changes, possibly induced by antibodies directed against the foreign *H-2* antigens during the period of background growth in the parental host, cannot be excluded.—J. Nat. Cancer Inst. 25:237–269, 1960.

TRACING INTERCELLULAR variability in tumors derived from isogenic-resistant (IR) mouse strains (*1*) with histocompatibility markers has been suggested as a possible approach to genetic studies on neoplastic cells (*2*). Klein and Klein (*3*, *4*) and Mitchison (*5*) have developed an experimental system for such analysis. They tested tumors originating in F_1 hybrids of 2 IR strains for their ability to give rise to "variant" sublines which could breed true in one or the other of the parental strains.

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[3] My sincere thanks to Professor G. Klein and Dr. E. Klein for their valuable advice.

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The IR strains used for the crosses were theoretically coisogenic, except for the histocompatibility-2 (*H-2*) locus. *H-2* differences are known to represent a strong barrier against the grafting of normal or neoplastic tissues. Progressive growth of a variant tumor subline in an otherwise *H-2*-incompatible parental strain therefore suggests a loss or masking of the *H-2*-determined isoantigens derived from the opposite parental strain (*4*). Model experiments (*3*) have shown that *H-2* compatible cells can be detected within a large population of incompatible cells by grafting the tissue on appropriate hosts.

Variant sublines are differentiated from "false positives" (*6*) by the criterion of selective compatibility with one parental strain to the exclusion of the other and unrelated strains (*4*). Parental-strain mice preimmunized against the opposite parental strain can be used to check variant compatibility in a more stringent way (*4*).

Klein and Klein (*4, 7–10*) and Mitchison (*11*) have found that it was possible to obtain variants from several F_1 tumors selectively compatible with one parental strain, while no variants specific for foreign genotypes were isolated. The variants were stable and did not revert to the original ype on prolonged passage in the F_1 genotype from which the tumor had been derived (*8*).

Klein and Klein (*7, 9*) showed that several tumors induced in a similar way gave rise to a characteristic and individually different frequency of variant sublines, indicating a biologic individuality of the different neoplasms.

Serologic tests showed that the variants had lost the ability to absorb or to induce the formation of hemagglutinins or cytotoxic antibodies directed against the *H-2* isoantigens derived from the opposite parental strain (*7–10, 12*).

Little is known about the mechanism of variant formation. Experiments by Bayreuther and E. Klein (*12*), involving the inoculation of a very small number of cells and the chromosomal examination of a methylcholanthrene-induced F_1 sarcoma and its 10 different maternal-strain variants, indicated that variants obtained from the same tumor on different occasions were mostly attributable to independent events. With the exception of 2 variants derived from the same original inoculum pool and showing closely identical karyotypes, all others were different from each other and from the original tumor in their detailed chromosomal cytology. Since chromosome numbers show considerable variation in primary methylcholanthrene-induced sarcomas (*13*), it is uncertain to what extent the chromosomal differences observed were directly related to the formation of variants; they may merely reflect the establishment of different cell clones on different occasions of variant formation.

The present investigation is a study of isoantigenic-variant formation in F_1 lymphomas, by serologic, chromosome cytologic, and transplantation methods. This material is particularly suitable, because lymphoma cells are sensitive to humoral isoantibodies *in vitro* in the presence of complement and can be tested directly for the presence of *H-2*-determined isoantigens

(*14*, *15*). The comparatively low degree of chromosomal variability in lymphomas makes them suitable for studies of the possible role of chromosomal changes in variant formation.

MATERIALS AND METHODS

Mice.—Four IR mouse strains were used: A/Sn (genotype *H-2^{a}H-2^a*), A.SW (genotype *H-2^{s}H-2^s*), A.BY (genotype *H-2^{b}H-2^b*), and A.CA (genotype *H-2^{f}H-2^f*). They were all developed by Snell (*1*) to introduce different *H-2* alleles on the common genetic background of the A/Sn strain. Skin-grafting experiments have shown that some non-*H-2* differences exist which separate the IR strains, but it was not possible to detect any such differences by tumor inoculations (*16*).[4] In addition, strains C3H/Kl (genotype *H-2^{k}H-2^k*), DBA/2/Kl (genotype *H-2^{d}H-2^d*), and C57BL/Kl (genotype *H-2^{b}H-2^b*) were used for some experiments. All strains were maintained by continuous single-line brother × sister mating. To keep the genetic background of the IR lines as similar as possible, A.SW, A.CA, and A.BY breeders were backcrossed to strain A mice after every fourth to fifth generation. The offspring were challenged with a tumor of A origin. Appropriate serologically typed survivors were subsequently selected to replace the breeding stock of the IR line.

Unless mentioned otherwise, animals of both sexes, 2 to 3 months of age, were used for all experiments. They were kept on a standard pellet diet and were given drinking water *ad libitum*. All experimental mice were earmarked at weaning and each genotype was given a characteristic mark.

Preimmunized mice were used in some experiments. They were inoculated with a tumor of the desired genotype 1 month before the actual experiment, except in those tests, described later, in which the mice were preimmunized with a mixture of normal kidney plus spleen and liver 7 days before the test.

Tumors.—Four lymphomas of homozygous and three of heterozygous F_1 hybrid origin derived from the IR stocks were studied. In addition, lymphomas of other genetic backgrounds, two of homozygous and two of heterozygous F_1 derivation were also investigated. Two of the heterozygous F_1-derived lymphomas in IR mice, LNSA and LNSB, originated in (A/Sn × A.SW)F_1 hybrids, while the third, LYCB, appeared in an (A.BY × A.CA)F_1 mouse. In animals implanted at the age of 1 month with a pellet containing 1 mg. stilbestrol plus 4 mg. cholesterol, all three neoplasms developed in 5 to 14 months. All other neoplasms developed spontaneously in old breeding females. The basic data on the tumors are shown in table 1.

Suspensions of all leukemic organs from the primary leukemic mice were transplanted subcutaneously into isologous recipients and gave rise

[4] Note added in proof: The origin of this heterozygosi has now been traced by Dr. Snell to a contamination of the A line used for his backcrosses during the early history of the IR lines (*35*).

to solid tumors at the site of inoculation. All subsequent passages were made from such solid tumors.

Tumor transplantation.—Aseptically removed tumors were brought into suspension by pressing them through a 60-mesh stainless-steel screen into sterile Ringer's solution containing 100 γ streptomycin and 100 I.U. penicillin per ml. The suspensions were inoculated subcutaneously into the right or left flank. Unless mentioned otherwise, the cell dose was 0.1 ml. of a suspension containing between 10^6 to 10^7 cells per ml. When known cell numbers were implanted, counts were made in a hemocytometer with 1:10 dilution of the cell suspension in Tyrode's solution containing 0.05 percent eosin, according to the procedure of Schrek (*17*). Red and pink cells were registered as dead, unstained cells as living.

The mice were inspected weekly for tumor development and were kept under observation for a minimum of 2 months after inoculation.

Serologic methods.—Antiserums were obtained from mice challenged with tumor tissue derived from hosts of various foreign *H-2* types. Four to five inoculations were given at intervals of about 10 days. Five to eight days after the last challenge the mice were bled to death from the retro-orbital sinus. Blood from 3 to 5 mice was pooled and allowed to clot at room temperature and was subsequently centrifuged at 1,000 $\times$ g for 20 minutes. The serums were collected and lyophilized in 0.05 ml. amounts and stored in a deepfreeze unit at $-15°$ C.

The antiserums were absorbed by incubation at 37° C. for 60 minutes, with equal volumes of packed cells from the liver, kidney, and spleen. The cells were washed three times with Ringer's solution and packed by centrifugation at 3,000 $\times$ g for 30 minutes. Two absorptions were usually performed; the mixture was then centrifuged and the antiserum lyophilized and stored.

Cytotoxic tests were made according to the method of Gorer and O'Gorman (*14, 15*). Cell suspensions were incubatad with antiserum and complement, and the proportion of viable cells was estimated before and after incubation by the supravital staining method of Schrek (*17*). Lymphoma cells and normal lymph-node cells were brought into suspension as described previously. All suspensions were washed once with Ringer's solution and resuspended to a final concentration of about 10^7 eosin-unstained cells per ml. Antiserums were diluted 1:5 with Ringer's solution, and then serial dilutions were made with a 1:5 mixture of normal mouse serum and Ringer's solution. In the controls the antiserums were replaced by normal mouse serums, diluted 1:5 in Ringer's solution. Guinea-pig serum was used as complement; it did not show any cytotoxic activity by itself. Equal volumes (0.05 ml.) of cell suspension, serially diluted isoimmune serum (1:5–1:320), and guinea-pig complement were mixed and incubated at 37° C. for 90 minutes. The percentage of eosin-unstained cells was determined before and after incubation. The first 300 cells were rapidly classified in each aliquot as stained and unstained.

Normal lymph-node cells of different genotypes and the lymphomas and their variant sublines were tested with antiserums containing isoanti-

bodies directed aginst various components of the *H-2* system. For each antiserum dilution a cytotoxic index was computed, defined as the difference between the percentage of unstained cells in the control and in the test suspension divided by the former figure. The titer was defined as the highest dilution of antiserum which still gave a cytotoxic index ≥ 0.15.

In another series of experiments lymphoma cells were treated *in vitro* with antiserum in the presence of complement, in an attempt to select antiserum-resistant cells. A mixture consisting of 0.2 ml. of a suspension containing 10^6 tumor cells plus 0.2 ml. guinea-pig complement plus 0.2 ml. 1:5 diluted antiserum was incubated at 37° C. for 3 hours. Normal serum from untreated mice was used for the controls. The number of eosin-unstained cells was determined according to the method of Schrek, after which a total of about 10^5 treated cells was inoculated subcutaneously into mice of the genotype of origin.

Cytologic methods.—The cytologic technique was a slight modification of the techniques described by Ford and Hamerton (*18*) and Bayreuther and E. Klein (*12*). Mice were given 2 intraperitoneal injections of 0.04 percent Colcemid (Ciba) solution in doses of 0.1 ml. per 10 gm. body weight, at an interval of 60 minutes. They were killed by cervical dislocation 60 minutes after the second injection. The tumors were excised and minced into small pieces. The material was incubated in a 1.12 percent sodium citrate solution for 15 to 25 minutes at 37° C., and then fixed in chilled 1:3 acetic acid-alcohol for 3 to 5 minutes, transferred to distilled water for 3 to 5 minutes, hydrolyzed in 1 N HCl at 60° C. for 8 to 10 minutes, and then stained with Feulgen reagent for 1 to 2 hours. Subsequently the material was transferred to SO_2 water for 3 minutes, and stored in 45 percent acetic acid in a −15° C. deepfreeze unit (*19*). Squash preparations were made with silicone-treated coverslips.

The slides were studied with a Zeiss standard phase-contrast microscope at a magnification of 1,500×. Metaphase figures with well-dispersed chromosomes and with intact outlines were selected for analysis. All chromosome counts were made on drawings prepared with an Abbe's drawing apparatus which permitted a fairly exact count. About 30 metaphase figures for each tumor were analyzed.

RESULTS

Transplantation Tests

The results of the transplantation tests are presented in detail in tables 2 to 10 and in text-figures 1 to 3. The main findings can be summarized as follows:

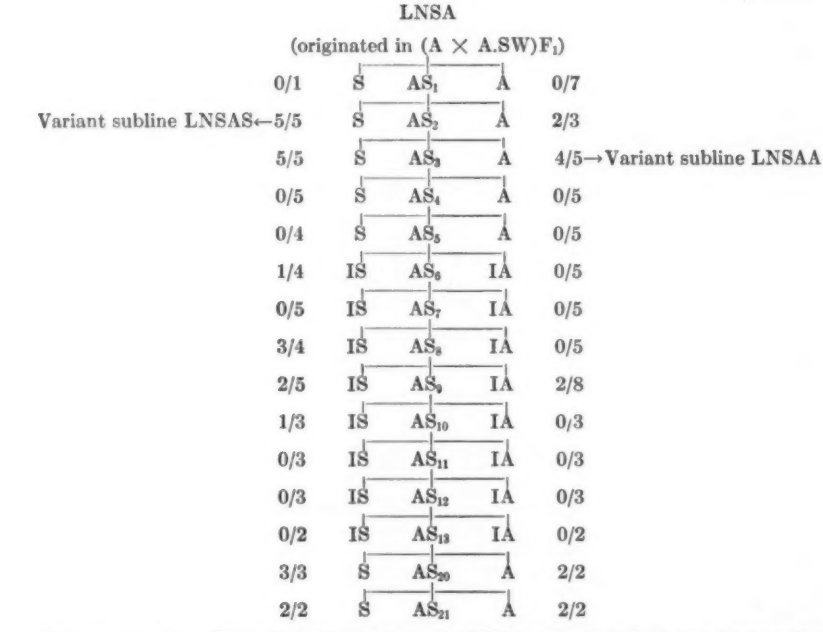
1) Inoculation of homozygous and heterozygous lymphomas into adult mice of different genotypes

Seven lymphomas which originated in IR mice with a common strain A background and 4 lymphomas derived from unrelated strains were

tested by transplantation into mice of various genotypes (tables 1, 2, and 3).

Both homozygous and heterozygous tumors grew progressively in most mice of the genotype of origin, while in other types the growth varied. The homozygous lymphomas did not grow in foreign strains. The heterozygous F_1 tumors gave rise to some progressively growing variant sublines in the parental strains of most mice. Such sublines were formed both from tumors arising in F_1 hybrids produced by outcrossing two IR lines (table 2) and from two unrelated strains (table 3).

The most extensive studies were made on LNSA and LNSB. Although both lymphomas were induced in (A × A.SW)F_1 hybrids by the same type of estrogen treatment, they behaved differently in transplantation tests. Thus LNSA grew in 12 of 32 strain A mice and 18 of 28 A.SW mice; LNSB grew in 2 of 87 strain A mice and 8 of 98 A.SW mice. It is apparent that A.SW was preferred to A by both tumors. In some passages LNSA took in both A and A.SW, but in other transfers it gave no takes at all (text-fig. 1). The available data do not permit definite conclusions about possible correlations between the formation of the 2 reciprocal parental variants from a given tumor. The percentage of takes in a parental strain decreased if the mice were preimmunized against the isoantigens of the


LNSA
(originated in (A × A.SW)F1)
0/1 S AS1 A 0/7
Variant subline LNSAS←5/5 S AS2 A 2/3
5/5 S AS3 A 4/5→Variant subline LNSAA
0/5 S AS4 A 0/5
0/4 S AS5 A 0/5
1/4 IS AS6 IA 0/5
0/5 IS AS7 IA 0/5
3/4 IS AS8 IA 0/5
2/5 IS AS9 IA 2/8
1/3 IS AS10 IA 0/3
0/3 IS AS11 IA 0/3
0/3 IS AS12 IA 0/3
0/2 IS AS13 IA 0/2
3/3 S AS20 A 2/2
2/2 S AS21 A 2/2

TEXT-FIGURE 1.—Transplantation history of LNSA, propagated during 21 transfers in (A × A.SW) F_1 hybrid mice. AS_1 = (A × A.SW)F_1 passage 1; A=A/Sn; S= A.SW; IA=A preimmunized against A.SW; IS = A.SW preimmunized against A. Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

TABLE 1.—Data on the primary hosts of the lymphomas investigated*

Tumor	Strain of origin	Sex	Age of mouse when killed (*months*)	Treatment	Thymus	Superficial lymph nodes	Mesenteric lymph nodes	Spleen	Liver	Kidney
SL1	A/Sn	♀	12	–	++	+	+	+	+	+
WL3	A.SW	♀	12	–	++	+	–	+	–	–
WL4	A.SW	♀	12	–	++	+	++	+	+	+
CCL1	A.CA	♀	12	–	++	–	–	–	–	–
LNSA	(A/Sn × A.SW)F_1	♂	6	†	+	+	+	+	–	–
LNSB	(A/Sn × A.SW)F_1	♂	7	†	+	+	+	++	++	–
LYCB	(A.BY × A.CA)F_1	♂	15	†	–	+	–	++	–	+
CL3	C3H	♀	12	–	–	+	+	+	–	–
DL1	DBA/2	♀	12	–	+	+	+	+	+	+
LHDA	(DBA/2 × C3H)F_1	♀	12	–	+	++	++	++	+	+
LHDB	(DBA/2 × C3H)F_1	♀	12	–	+	+	–	++	+	–

*The pathologic anatomic classification is based on gross examination: – = normal, + = moderate, and ++ = excessive organ enlargement due to leukemic infiltration.
†A pellet containing 1 mg. stilbestrol plus 4 mg. cholesterol was implanted subcutaneously in mice at the age of 1 month.

TABLE 2.—Pooled results of transplantation tests with homozygous and heterozygous lymphomas originating in various IR mice with strain A background and their derived variant sublines

Tumor	Strain of origin	Total No. of passages	Host*											
			(A × A.SW)F$_1$	A/Sn	A.SW	A.CA	A.BY	(A.CA × A.BY)F$_1$	A/Sn preimmunized with A.SW tissue	A.SW preimmunized with A/Sn tissue	A.CA preimmunized with A.BY tissue	DBA/2	DBA/2 preimmunized with A × A.SW tissue†	C3H
SL1	A	7		21/23	0/29	0/4	0/6	0/2						
WL3	A.SW	5	5/5	0/10	27/27		0/2							
WL4	A.SW	8	8/8	0/10	22/22	0/4	0/4	0/6						
CCL1	A.CA	7		0/11	0/9	19/19	0/19							
LNSA	(A × A.SW)F$_1$	21	52/53	12/32	18/28				2/38	10/33		9/9	2/5	0/4
LNSAA	(A × A.SW)F$_1$‡	14 in A	7/7	15/16	2/2				29/40§	6/40				
LNSAS	(A × A.SW)F$_1$‡	15 in A.SW	5/5	14/14	12/13				1/31	42/54		0/4		0/3
LNSB	(A × A.SW)F$_1$	30	71/72	2/87	8/98									
LNSBS	(A × A.SW)F$_1$‡	11 in A.SW	6/6	0/22	11/12				0/3	18/22‖				
LNSBA	(A × A.SW)F$_1$‡	6 in A	2/2	8/8	0/10				5/13	0/2				
LYCB	(A.CA × A.BY)F$_1$	10				2/16	0/22	22/26						
LYCBC	(A.CA × A.BY)F$_1$	5 in A.CA				8/9	0/10	2/2			9/9			

*Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

†The mice were given 1 challenge dose with LNSA, irradiated with 5000 r *in vitro*.

‡

Variant:	Selected from:	Host of selection:
LNSAA	LNSA, 3d transfer	A
LNSAS	LNSA, 2d transfer	A.SW
LNSBA	LNSB, 21st transfer	A
LNSBS	LNSB, 3d transfer	A.SW
LYCBC	LYCB, 3d transfer	A.CA

§One tumor from the eighth passage killed all 3 strain A mice preimmunized 7 days before grafting against A.SW normal tissue.

‖One tumor from the fifth passage killed all 3 A.SW mice preimmunized 7 days before grafting against strain A normal tissue.

TABLE 3.—Pooled results of transplantation tests on homozygous and heterozygous lymphomas originating in DBA/2, C3H, and (DBA/2 × C3H)F_1 mice and their derived variant sublines

Tumor	Strain of origin	Total No. of passages	Host*						
			(DBA/2 × C3H)F_1	DBA/2	C3H	C57BL	ABY	DBA/2 preimmunized against C3H	C3H preimmunized against DBA/2
CL3	C3H	5		0/11	24/27				
DL1	DBA/2	5		25/25	0/2		0/4		
LHDA	(DBA/2 × C3H)F_1	2	10/10	0/4	1/3				
LHDAH	(DBA/2 × C3H)F_1†	5 in C3H	5/5	0/6	13/13	0/2			4/5
LHDB	(DBA/2 × C3H)F_1	5	12/12	3/11	2/11	0/2			
LHDBD	(DBA/2 × C3H)F_1†	2 in DBA/2	2/3	11/11	0/8			2/2	

*Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

†Variant: Selected from: Host of selection:

LHDAH LHDA, 1st transfer C3H

LHDBD LHDB, 3d transfer DBA/2

other strain; thus LNSA grew in 10 of 33 A.SW mice and 2 of 38 A mice, under these conditions. A somewhat higher number of takes in the parental strains was obtained during the later transfers of the tumors. Thus 6 of 42 A.SW mice and 2 of 40 A mice inoculated with LNSB developed tumors in the second 15 passages (16–30), in comparison with the 2 of 56 and none of 47 mice, respectively, which developed during the first 15 transfers (table 4).

TABLE 4.—Progressively growing takes in parental strains after inoculation with LNSB from different transfer generations of (A × A.SW)F_1 hybrid mice

Results pooled from passages	Host*		
	(A × A.SW)F_1	A	A.SW
1–5	14/14	0/24	1/26
6–10	7/8	0/10	0/16
11–15	10/10	0/13	1/14
Total	31/32	0/47	2/56
16–20	10/10	0/9	3/16
21–25	20/20	1/22	2/16
26–30	10/10	1/9	1/10
Total	40/40	2/40	6/42

*Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

Except for occasional takes in parental-strain mice, all F_1 lymphomas, except one, were specific in their transplantability and grew only in their F_1 genotype of origin. LNSA grew indiscriminately in nonimmunized mice of both parental strains and also in some foreign genotypes such as DBA/2, in which it grew in 100 percent of the mice. The incidence of takes of LNSA was reduced by the use of preimmunized hosts (table 2).

Occasional takes of F_1 lymphomas in parental-strain mice were tested further to see whether they could breed true selectively in the same parental strains but not in other strains. If this occurred, they were called "variants." Two variants were established from LNSA: LNSAA and LNSAS. They were taken from A and A.SW hosts, respectively, in which they arose after the hosts had been inoculated with LNSA from its second and third passage, respectively. One variant subline of LNSB, LNSBS, was established in A.SW from the third passage and another variant LNSBA was established in A from the 21st passage of the lymphoma. LYCB gave rise to one variant, LYCBC, in A.CA after its third passage. LHDAH was isolated in C3H from the first transfer of LHDA, and LHDBD in DBA/2 from the third passage of LHDB.

2) *Transplantation tests on variant sublines*

The variant sublines isolated from the various F_1 tumors were tested by serial transplantation into untreated and preimmunized mice of different genotypes (tables 2, 3, 5, and 6).

TABLE 5.—Transplantation tests on variant subline LNSAA obtained from the third passage of LNSA in an untreated A mouse and carried subsequently in A hosts

	Host*				
Passage in A	(A × A.SW)F_1	A	A.SW	A preimmunized with A.SW tissue	A.SW preimmunized with A tissue
1		3/3		0/3	0/3
2	3/3	3/3		0/3	2/5
3		2/3		0/4	3/4
4		3/3		4/4	0/3
5				5/5	0/4
6				3/3	0/4
7				5/5	1/3
8	2/2			3/3	0/3
9	2/2	2/2	2/2	2/2	
10				2/2	0/2
11				1/2	0/2
12		2/2			0/2
13				2/2	0/3
14				2/2	0/2
Total:	7/7	15/16	2/2	29/40	6/40

*Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

LNSAA (tables 2 and 5) grew in both A and A.SW mice if the hosts were untreated. In preimmunized hosts, there was a change in its transplantation behavior after the first 3 transfers. During the first 3 passages LNSAA grew in 5 of 12 A.SW mice preimmunized against A and in none of 10 A mice preimmunized against A.SW, and was therefore propagated in untreated A hosts. Starting with the fourth transplant generation, LNSAA also grew in preimmunized A mice. Concomitantly with this change, it lost the ability to kill preimmunized A.SW mice. Subsequently it was propagated in A mice preimmunized against A.SW. It killed 29 of 30 such hosts between passages 4 to 14. During the same period, LNSAA killed only 1 of 28 A.SW mice preimmunized against A.

LNSAS (table 2) grew in both A.SW and A mice if the hosts were untreated. It could also grow immediately in preimmunized A.SW hosts, and was continuously propagated there. On the whole, 42 of 54 A.SW mice preimmunized against A and 1 of 31 A mice preimmunized against A.SW succumbed to progressively growing tumors. Doses up to 10^6 LNSAS cells from the tenth A.SW transfer were inoculated in DBA/2 mice and did not grow in any of 4 mice receiving an injection.

LNSBA was similar to LNSAA since it killed only 1 of 7 preimmunized A mice during its first 3 transfers (table 6). Between the 4th and the 6th passages it also killed 4 of 6 A mice preimmunized against A.SW but did not grow in 10 A.SW mice.

LNSBS, tested for 11 consecutive passages, grew in 11 of 12 untreated A.SW mice, 18 of 22 A.SW mice preimmunized against A, and in none of 22 untreated A mice (table 2). It grew in preimmunized A.SW mice from the first transfer and was therefore carried in these mice.

LYCBC was tested for 5 passages and grew in 8 of 9 untreated A.CA mice, 9 of 9 A.CA mice preimmunized against A.BY, and in none of 10

TABLE 6.—Transplantation tests on variant subline LNSBA obtained from the 21st passage of LNSB in an untreated A mouse and carried subsequently in A hosts

Passage in A	Host*				
	(A × A.SW)F_1	A	A.SW	A preimmunized with A.SW tissue	A.SW preimmunized with A tissue
1	2/2	2/2	0/2	0/3	0/2
2		2/2		1/2	
3		2/2	0/2	0/2	
4		2/2	0/2	1/2	0/2
5		2/2	0/2	2/2	
6			0/2	1/2	

*Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

untreated A.BY mice (table 2). It was carried in preimmunized A.CA mice after the first transfer.

Only preliminary tests are available on the variants obtained from F_1-hybrid tumors arising in genotypes produced by the outcrossing of 2 unrelated mouse strains.

LHDAH grew in 13 of 13 untreated C3H mice. It was propagated in preimmunized C3H mice from its second passage (table 3), and did not grow in DBA/2 or C57BL mice.

LHDBD, which was isolated from a DBA/2 mouse, grew in 11 of 11 untreated mice and 2 of 2 preimmunized DBA/2 mice (table 3). It did not grow in C3H.

3) *Transplantation of known number of LNSA cells to preimmunized mice of different genotypes*

In a series of experiments different cell doses of LNSA from the 21st transfer generation were tested in preimmunized mice (table 7). Takes were obtained from doses between 10^5 and 5×10^7 in some preimmunized A.SW, (A.SW × A.CA)F_1, and (A.SW × A.BY)F_1 mice. Only 2 of 26 A

TABLE 7.—Subcutaneous inoculation of a known number of LNSA cells from the 21st transfer into mice of different genotypes preimmunized with one challenge dose of incompatible tumor tissue, about 1 month before the experiment

Number of unstained cells inoculated	Host*				
	A preimmunized with A.SW tissue	(A × A.BY)F_1 preimmunized with A.SW tissue	A.SW preimmunized with A tissue	(A.SW × A.BY)F_1 preimmunized with A tissue	(A.SW × A.CA)F_1 preimmunized with A tissue
10		0/3			0/3
10^2	0/4			0/2	0/2
10^4	1/4		0/2		0/2
10^5	0/4		2/2	1/2	
10^6	0/5		1/5		
10^7	0/5		1/3	2/2	
5×10^7		1/1			1/1
Total	1/22	1/4	4/12	3/6	1/8

*Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

[or (A × A.BY)F_1] mice developed tumors. No good correlation was found between cell dose and growth of tumor in preimmunized mice.

4) *Propagation of a variant isolated from one parental strain in mice of the opposite parental strain*

An attempt was made to "switch" a variant selected in and compatible with preimmunized mice of 1 parental strain so that it would also grow in preimmunized mice of the opposite parental strain. The 2 LNSA variants, LNSAA and LNSAS, were chosen for these experiments because they both grew in almost 100 percent of untreated mice of both parental strains, but when preimmunized hosts were used, they were entirely restricted to the parental strain from which they were selected.

LNSAA, capable of growing in preimmunized A, was carried for 12 passages in untreated A.SW mice without any change in its transplantation behavior (table 8). It maintained its ability to grow in a high percentage of preimmunized A mice, but grew only in 2 of 27 preimmunized A.SW mice.

TABLE 8.—Results of transplantation tests on LNSAA, selected during 8 passages in A mice and propagated for 12 transfer generations in untreated A.SW mice

Transfer generations in A.SW	Host*				
	(A × A.SW)F_1	A.SW	A	A preimmunized with A.SW tissue	A.SW preimmunized with A tissue
1	2/2	3/3	3/3		0/3
2		3/3		2/2	0/2
3		2/2		1/2	1/2
4		2/2		2/2	0/3
5		2/2		2/2	0/2
6		2/2	2/2		0/2
7	2/2	2/2		2/2	0/2
8	2/2	2/2		2/3	0/2
9		2/2		2/2	0/2
10		1/1		3/3	0/3
11		2/2		2/2	0/1
12		2/2		2/2	1/3
Total	6/6	25/25	5/5	18/20	2/27

*Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

LNSAS, capable of growing in preimmunized A.SW mice, was carried for 17 passages in untreated A mice, in which it grew in all mice inoculated but did not change (table 9). Its ability to grow in preimmunized A.SW mice remained high and only 2 of 37 preimmunized A hosts were killed by a tumor.

5) *Transplantation to newborn mice*

LNSB samples from the eleventh to seventeenth passage in (A × A.SW)F_1 were inoculated into newborn mice, less than 24 hours old, of the genotypes A, A.SW, (A.SW × DBA/2)F_1, and (A.SW × C3H)F_1 (text-fig. 2). Most mice developed progressively growing tumors, and

TABLE 9.—Results of transplantation tests on LNSAS, selected during 10 passages in A.SW mice and propagated for 17 transfer generations in untreated A mice

Transfer generations in A	Host*			
	(A × A.SW)F_1	A	A preimmunized with A.SW tissue	A.SW preimmunized with A tissue
1		2/2	1/2	3/3
2		2/2	0/3	2/2
3		3/3	0/2	2/2
4		2/2	0/2	3/3
5		2/2	0/2	2/2
6		2/2	0/3	2/2
7	2/2	2/2	0/2	3/3
8	2/2	2/2	0/2	1/2
9		1/2	0/2	1/2
10		2/2	0/2	2/2
11		2/2	0/2	2/2
12		2/2	1/2	2/2
13		2/2	0/3	1/2
14		2/2	0/2	2/2
15		2/2	0/2	2/2
16		2/2	0/2	1/2
17		2/2	0/2	2/2
Total	4/4	34/35	2/37	33/37

*Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

these were tested further by transplantation to adult mice of appropriate genotypes.

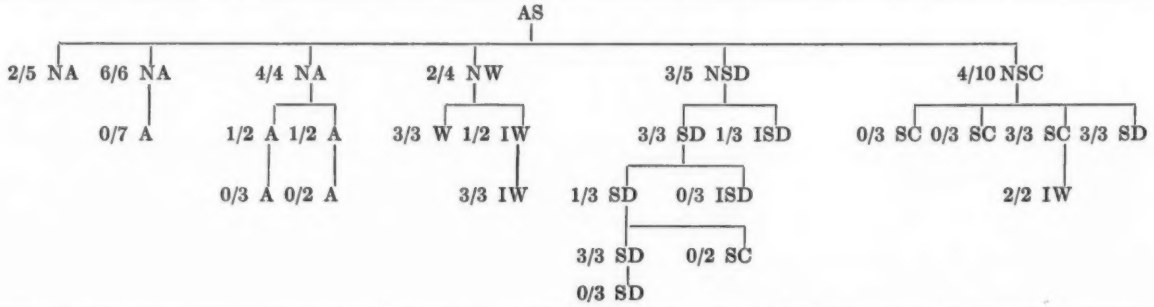
After passage through newborn A.SW mice, the tumor behaved as an A.SW variant. However it was not possible to obtain any variants of A mice in this way, though 3 trials were made.

In another series of experiments LNSB was inoculated into newborn (A.SW × DBA/2)F_1 and (A.SW × C3H)F_1 mice. The tumors obtained were tested to see whether they were different from each other and from ordinary A.SW variants (table 10). DBA/2 and C3H mice contain the isoantigenic factors D and K respectively, which are parts of the isoantigenic complex differentiating A and A.SW (*20*). It is conceivable that a variant in A.SW × C3H or in A.SW × DBA/2 would have lost only one of the isoantigenic components.

It was not possible to obtain any variants growing selectively in A.SW × DBA/2. One subline isolated in this genotype grew only in 1 of 3 preimmunized A.SW × DBA/2 mice, during its first transfer, and later died out in nonimmunized A.SW × DBA/2 mice (text-fig. 2, table 10). It did not grow in A.SW × C3H.

The subline isolated in newborn A.SW × C3H mice grew directly in adults of A.SW × C3H, A.SW × DBA/2, and A.SW, even if they were preimmunized against A, suggesting that both the isoantigenic components D and K had been lost from this subline.

In another series of experiments LNSAS was inoculated into newborn A.BY mice and grew in 1 of 5 (text-fig. 3). This tumor was further transplanted to adult A.BY mice and could kill these mice after about 3 months,



NA = newborn A
NW = newborn A.SW
NSD = newborn A.SW × DBA/2
NSC = newborn A.SW × C3H
A = adult A
W = adult A.SW
AS = adult(A × A.SW)F_1
SD = adult(A.SW × DBA/2)F_1
SC = adult(A.SW × C3H)F_1
IW = adult A.SW preimmunized against A tissue
ISD = adult A.SW × DBA/2 preimmunized against A tissue

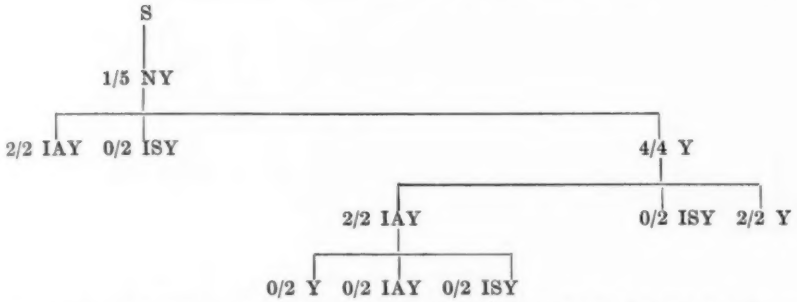
Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

TEXT-FIGURE 2.—Passage of LNSB through newborn mice of various genotypes (samples taken from the 11 to 17th passage in (A × A.SW)F_1 hybrid mice

TABLE 10.—Pooled transplantation results with 2 sublines of LNSB transferred in (A.SW × DBA/2)F_1 and (A.SW × C3H)F_1 mice, respectively

Genotype of origin	Genotype of selection	Number of passages	Host*							
			(A/Sn × A.SW)F_1	(A.SW × DBA/2)F_1	(A.SW × C3H)F_1	A/Sn	A.SW	(A.SW × DBA/2)F_1 preimmunized against A/Sn	(A.SW × C3H)F_1 preimmunized against A/Sn	A.SW preimmunized against A/Sn
(A/Sn × A.SW)F_1	(A.SW × DBA/2)F_1	4	2/2	7/12	0/5	0/8	4/8	1/10	–	0/3
(A/Sn × A.SW)F_1	(A.SW × C3H)F_1	5	5/6	11/11	12/12	0/9	7/7	7/7	2/2	7/9

*Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.



TEXT-FIGURE 3.—Transplantation results of a subline of LNSAS isolated from a newborn A.BY mouse and further tested in A.BY. Fractions indicate the number of mice killed by progressively growing tumors over total number inoculated.

S = adult A.SW

NY = newborn A.BY

Y = adult A.BY

IAY = adult A.BY preimmunized with A tissue

ISY = adult A.BY preimmunized with A.SW tissue

an approximately fourfold prolongation of survival when compared to a compatible host. If the A.BY mice were preimmunized against strain A, LNSAS grew still more slowly and did not grow at all if the mice were preimmunized against A.SW. Similar results were obtained during the next transfer. After these two passages in adult A.BY mice the tumor subline did not grow progressively in A.BY.

Serologic Tests

The results of the serologic tests are presented in tables 11 to 16 and in text-figure 4. The main findings will be summarized here.

Three homozygous lymphomas, SL1, WL3, and WL4, and 4 heterozygous F_1 lymphomas, LNSA, LNSB, LYCB, and LHDB, were tested for their sensitivity to *H-2* isoantibodies, by the technique of Gorer and O'Gorman. The cytotoxic index at given antiserum dilutions and the titer of the tumors were compared. The strength of the antiserums was tested against normal lymph-node cells so that different serums could be compared. For testing heterozygous F_1 lymphomas, serums of similar strength were chosen with regard to the two parental strain components. Generally, serums produced by a similar series of treatment in A against A.SW mice and *vice versa* had a similar strength.

All lymphomas were highly sensitive to the action of isoantiserum in the presence of complement, up to a titer of 1:40 to 1:160 (table 11). There was no significant difference between the sensitivity of lymphoma cells and normal lymph-node cells (text-fig. 4). The heterozygous lymphomas were affected to a similar extent by isoantibodies directed against either parental strain. Thus LNSB cells were killed at a titer of 1:40 to 1:80 by both A.SW anti-A and A anti-A.SW serums.

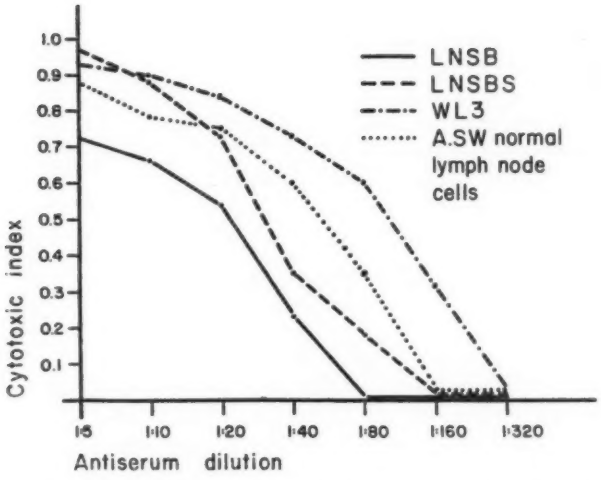
The source of error by contaminating stroma cells of the host seems to have little importance for the serologic results, since suspensions of

TABLE 11.—Sensitivity of different homozygous and heterozygous lymphomas and their variant sublines toward *H-2* isoantiserums

Tumor						Antiserum			
Name	Passages tested	Genotype of origin	Genotype of selective transfer	Last host	Ability of variant subline to grow in preimmunized mice	Prepared in	Against *	Cytotoxic index at the antiserum dilution 1:10 (*mean*)	Reaction titer
SL1	1, 3	A	—	A	−	A.SW	A[1]	0. 88	1:80
SL1	4	A	—	A × A.SW F_1	−	A.SW	A[3]	0. 90	1:80
SL1	4	A	—	A × A.SW F_1	−	A	A.SW[4]	—	Negative
WL3	5	A.SW	—	A.SW	−	A	A.SW[2]	0. 92	1:160
WL4	3	A.SW	—	A.SW	−	A	A.SW[2]	0. 84	1:160
LNSA	1, 4, 10, 24	A × A.SW F_1	—	A × A.SW F_1	−	A.SW	A[1,3]	0. 59	1:40
LNSA	1, 4, 10, 24	A × A.SW F_1	—	A × A.SW F_1	−	A	A.SW[2,4]	0. 53	1:40
LNSA	22	A × A.SW F_1	—	DBA/2	−	A.SW	A[3]	0. 60	1:40
LNSA	22	A × A.SW F_1	—	DBA/2	−	A	A.SW[4]	0. 60	1:40
LNSAA	1	A × A.SW F_1	A	A (untreated)	−	A.SW	A[1]	0. 80	1:80
LNSAA	1	A × A.SW F_1	A	A (untreated)	−	A	A.SW[2]	0. 78	1:80
LNSAA	5	A × A.SW F_1	A	A (preimmunized)	+	A.SW	A[1]	0. 70	1:80
LNSAA	5	A × A.SW F_1	A	A (preimmunized)	+	A	A.SW[2]	0	Negative
LNSAS	1	A × A.SW F_1	A.SW	A.SW (preimmunized)	+	A	A.SW[2]	0. 87	1:80
LNSAS	1	A × A.SW F_1	A.SW	A.SW (preimmunized)	+	A.SW	A[1]	0	Negative
LNSAS	3	A × A.SW F_1	A.SW	A × A.SW F_1	+	A	A.SW[2]	0. 82	1:40
LNSAS	3	A × A.SW F_1	A.SW	A × A.SW F_1	+	A.SW	A[1]	0.	Negative
LNSAS	6	A × A.SW F_1	A.SW	A.BY	+	A	A.SW[2]	0. 91	1:40
LNSAS	6	A × A.SW F_1	A.SW	A.BY	+	A.SW	A[1]	0	Negative
LNSAS	7, 10	A × A.SW F_1	A.SW	A	+	A	A.SW[2,4]	0. 80	1:80
LNSAS	7, 10	A × A.SW F_1	A.SW	A	+	A.SW	A[1,3]	0	Negative
LNSB	1, 6, 13, 24	A × A.SW F_1	—	A × A.SW F_1	−	A	A.SW[2,4]	0. 61	1:40
LNSBS	1, 6, 13, 24	A × A.SW F_1	—	A × A.SW F_1	−	A.SW	A[1,3]	0. 69	1:40
LNSBA	1	A × A.SW F_1	A	A (untreated)	−	A.SW	A[3]	0. 98	1:160
LNSBA	1	A × A.SW F_1	A	A (untreated)	−	A	A.SW[4]	0. 55	1:40
LNSBA	5	A × A.SW F_1	A	A (preimmunized)	+	A.SW	A[3]	0. 92	1:80

LNSBA	5	A × A.SW F_1	A	A (preimmunized)	+	A	A.SW[4]	0	Negative
LNSBS	1	A × A.SW F_1	A.SW	A.SW (untreated)	+	A.SW	A[2]	0	Negative
LNSBS	1	A × A.SW F_1	A.SW	A.SW (untreated)	+	A	A.SW[1]	0. 87	1:40
LNSBS	1, 2, 3	A × A.SW F_1	A.SW	A.SW (preimmunized)	+	A.SW	A[2]	0	Negative
LNSBS	1, 2, 3	A × A.SW F_1	A.SW	A.SW (preimmunized)	+	A	A.SW[1]	0. 92	1:40
LYCB	4	A.CA × A.BY F_1	—	A.CA × A.BY F_1	−	A	A.CA[5]	0. 80	1:40
LYCB	4	A.CA × A.BY F_1	—	A.CA × A.BY F_1	−	A.CA	A[6]	0. 53	1:40
LYCBC	1	A.CA × A.BY F_1	A.CA	A.CA (preimmunized)	+	A	A.CA[5]	0. 87	1:80
LYCBC	1	A.CA × A.BY F_1	A.CA	A.CA (preimmunized)	+	A.CA	A[6]	0	Negative
LYCBC	1	A.CA × A.BY F_1	A.CA	A.CA (untreated)	+	A	A.CA[3]	0. 83	1:80
LYCBC	1	A.CA × A.BY F_1	A.CA	A.CA (untreated)	+	A.CA	A[6]	0	Negative
LHDB	3	DBA/2 × C3H F_1	—	DBA/2 × C3H F_1	−	A.SW	A[3] †	0. 50	1:20
LHDB	3	DBA/2 × C3H F_1	—	DBA/2 × C3H F_1	−	A.SW	A[3] ‡	0. 62	1:20
LHDBD2	1	DBA/2 × C3H F_1	DBA/2 × A.SW F_1	DBA/2 × A.SW F_1	+	A.SW	A[3] †	0	Negative
LHDBD2	1	DBA/2 × C3H F_1	DBA/2 × A.SW F_1	DBA/2 × A.SW F_1	+	A.SW	A[3] ‡	0. 60	1:20
LHDBD2	1	DBA/2 × C3H F_1	DBA/2 × A.SW F_1	DBA/2 × A.SW F_1	+	A	A.SW[4]	0. 12	Negative

* Superscript 1, 2, 3, 4, 5, and 6 indicate the 6 pools from which the isoantiserum was taken.
† Absorbed with DBA/2 normal tissue.
‡ Absorbed with C3H normal tissue.



TEXT-FIGURE 4.—Cytotoxic effect of an antiserum, prepared in A against A.SW tissue, on normal A.SW lymph-node cells and on cells from lymphomas WL3, LNSB, and the variant subline LNSBS.

SL1 taken from an A × A.SW host, and LHDBD2, taken from an DBA/2 × A.SW host, were both resistant to the action of A anti-A.SW serums.

The 3 parental variants, LNSAA, LNSAS, and LNSBS, were tested against *H-2* isoantiserums directed against both parental components entering the original F_1 cross. All variants capable of growing in preimmunized mice of the parental strain in which they had been selected had lost their sensitivity toward antibodies directed against the opposite parental strain. This loss was not reversible by returning the variants to their original F_1 genotype for passage or by making them grow in a foreign strain such as A.BY. "False positives" of LNSA appearing in DBA/2, but incompatible with preimmunized mice, were unchanged in their sensitivity to isoantibodies directed against either parental strain.

An attempt was made to study whether isoantigenic gains occurred during the variant formation either by incorporation of determinants from the parental host or by mutation to alternative isoantigens. Serologic tests were made on LHDBD2 derived from LHDB originating in a (DBA/2 × A.SW)F_1 hybrid, to see if the variant subline possessed A.SW isoantigens. No such antigens were found, since the variant was resistant to A-anti-A.SW antibodies.

All A.SW variants examined and the A.CA variant isolated from LYCB had completely lost the sensitivity to antibodies directed against the opposite parent. The A.SW variants were also immediately compatible with preimmunized A.SW mice after their first cycle of growth in A.SW. A more complicated picture was presented when the two A variants, LNSAA and LNSBA, were studied. These variants could not grow in

preimmunized A mice until the fourth transfer generation. LNSAA, tested serologically after the first transfer, was similar to the original LNSA tumor in that it was sensitive to antibodies against both parental strains. The case with LNSBA was similar though this variant showed a decreased sensitivity to anti-A.SW antibodies, as shown by both the titer and the cytotoxic index. When the variants were tested after 4 transfer generations in nonimmunized strain A mice, and then also grew in A mice, preimmunized against A.SW, they had lost their sensitivity to A-anti-A.SW antibodies.

Serologic tests were also performed on sublines obtained from LNSB in (A.SW × DBA/2)F_1 and in (A.SW × C3H)F_1 mice (table 12). Theoretically an A tumor would be expected to lose the two *H-2* isoantigens D and K, which are present in DBA/2 and C3H, respectively (*20*), to become compatible with A.SW. In agreement with the transplantation findings on this material, however, no monofactorial losses of isoantigens were found. The tumor line in A.SW × DBA/2 was sensitive to A.SW-anti-A serum, even after absorption with DBA/2 or C3H tissues. It was thus similar to the original LNSB line, in that it did not grow in preimmunized (A.SW × DBA/2)F_1 mice. The A.SW × C3H line was resistant even to unabsorbed A.SW-anti-A serum, indicating, similar to the LNSBS variant, a loss of both D and K, since it could grow in an A.SW host preimmunized against A.

Furthermore serologic tests were performed (table 13) on the LNSAA and LNSAS variants which had been propagated for 12 consecutive passages in untreated A.SW mice and for 17 transfer generations in untreated A mice [*cf.* 4), "Transplantation Tests"]. The tumors were returned to the original (A × A.SW)F_1 genotype for 1 generation before their sensitivity to A.SW-anti-A and A-anti-A.SW serums was tested. There was no change in the antibody sensitivity of the cells after these transfers. Serologically LNSAS thus remained an A.SW variant and LNSAA an A variant, despite the effort to "switch" them over to the opposite type.

A comparison between different tests indicates that heterozygous lymphomas are less sensitive to a given antiserum than homozygous

TABLE 12.—Serologic analysis of LNSB sublines carried in (A.SW × DBA/2)F_1 and (A.SW × C3H)F_1 mice respectively

Host of origin	Host of selection	Passage	Antiserum: Prepared in	Against	Absorbed with	Cytotoxic index at antiserum dilution 1:10	Reaction titer
(A × A.SW)F_1	(A.SW × DBA)F_1	2	A.SW	A	—	0.9	1:80
					DBA/2	0.5	1:20
					C3H	0.6	1:20
			A	A.SW	—	0.9	1:80
	(A.SW × C3H)F_1	3	A.SW	A	—	0	Negative
			A	A.SW	—	0.70	1:80

TABLE 13.—Results of serologic tests on LNSAA and LNSAS carried in untreated mice of the opposite parental strain in which these variants were originally selected

Tumor				Antiserum		Percent of unstained cells			
Name	Selected for growth in	Propagated subsequently in	Number of passages in strains of mice in column 3	Prepared in	Against	Control	Antiserum 1:5	Cytotoxic index at antiserum dilution 1:5	Reaction titer
LNSAA	A (8 transfers)	A.SW	7	A.SW	A	63	10	0. 84	1:80
				A	A.SW	63	68	0	Negative
			8	A.SW	A	65	1	0. 98	1:160
				A	A.SW	65	56	0. 14	Negative
			9	A.SW	A	46	5	0. 89	1:80
				A	A.SW	46	41	0. 11	Negative
LNSAS	A.SW (8 transfers)	A	7	A.SW	A	73	75	0	Negative
				A	A.SW	73	27	0. 63	1:80
			9	A.SW	A	50	52	0	Negative
				A	A.SW	50	12	0. 76	1:20
			12	A.SW	A	52	45	0. 13	Negative
				A	A.SW	52	1	0. 98	1:80

lymphomas are or than the corresponding parental-strain variants of the F_1 lymphomas themselves. To study this more critically, comparative tests were made with the same pool of isoantiserums and complement (table 14). When the lymphomas LNSA, LNSB, SL1, WL3, and the variant sublines LNSAA, LNSAS, and LNSBS were tested in this way, it appeared that the homozygous tumors and the parental variants had a slightly but consistently higher sensitivity than the heterozygous F_1 lymphomas, manifested by a somewhat higher cytotoxic index and by a higher titer. A representative case is shown in text-figure 4, indicating that the LNSB lymphoma was somewhat less sensitive to treatment with A-anti-A.SW serum than either its A.SW variant, LNSBS, or the homozygous WL3 lymphoma.

No significant differences were found in the sensitivity of LNSA and LNSB against isoantiserum, though the transplantation behavior of the tumors was clearly different.

Repeated antiserum treatments of LNSA in vitro

An attempt was made to select parental variants from LNSA by *in vitro* incubation of lymphoma cells with isoantiserum followed by transplantation to the original $(A \times A.SW)F_1$ hybrid type (table 15). The treatments were performed like the ordinary cytotoxic tests. Afterward the number of cells unstained by eosin was determined; between 51 and 94 percent of the cells were stainable.

When 10^5-treated cells were inoculated, they developed into tumors after about 1 month, and formed 2 sublines of LNSA: LNSA-IVA and LNSA-IVS. Each was treated with the appropriate anti-A.SW and anti-A serum, respectively, before each serial transplantation. After 4 such passages the cell populations were still unchanged in their sensitivity to anti-A or anti-A.SW antibodies.

Cytologic Studies of Chromosomes

A cytologic study of chromosomes was made on a few samples of LNSA, LNSB, and their variant sublines. The results are shown in table 16. They represent findings on subcutaneous tumors appearing at the inoculation site. In one case of generalized leukemia, mesenteric lymph nodes were studied (LNSA, generation 6) and the same stem line was found as in the subcutaneous tumors.

In all populations the predominant cell type had 41 chromosomes and no typical markers. There were no significant differences in the variability of chromosome numbers in different tumors nor any significant differences between the idiograms of an LNSB tumor and its derived A.SW subline, LNSBS. Two typical metaphase plates are shown in text-figures 5A and 5B.

TABLE 14.—Comparison of different lymphomas and normal lymph-node cells for their sensitivity toward the same isoantiserum pools

Cells	Number of passage	Strain of origin	Host	Antiserum: Prepared in	Antiserum: Against*	Percent of unstained cells: Control	Percent of unstained cells: Treated with antiserum 1:5	Cytotoxic index 1:5	Final titer
Normal lymph-node cells	—	A	A	A.SW	A	58	5	0.91	1:80
Normal lymph-node cells	—	A.SW	A.SW	A	A.SW	62	8	0.88	1:80
SL1	3	A	A.SW	A.SW	A	74	7	0.90	1:80
WL3	5	A.SW	A.SW	A	A.SW	76	5	0.93	1:160
LNSA	24	(A × A.SW)F1	(A × A.SW)F1	A.SW	A	87	16	0.82	1:40
				A	A.SW	87	18	0.79	1:40
LNSAA†	3 in A × A.SW, 5 in A	(A × A.SW)F1	(A × A.SW)F1	A.SW	A	55	7	0.88	1:80
				A	A.SW	55	51	0	Negative
LNSAS‡	2 in A × A.SW, 10 in A	(A × A.SW)F1	(A × A.SW)F1	A.SW	A	58	59	0	Negative
				A	A.SW	58	5	0.91	1:80
LNSB	24	(A × A.SW)F1	(A × A.SW)F1	A.SW	A	49	18	0.63	1:40
				A	A.SW	49	13	0.73	1:40
LNSBS‡	3 in A × A.SW, 3 in A.SW	(A × A.SW)F1	A.SW	A.SW	A	48	2	0.96	1:80
				A	A.SW	48	52	0	Negative

*Antiserum against A was produced by giving A.SW mice 5 challenges with A tumors at intervals of 10 days; the antiserum was taken 5 days after the last challenge. Antiserum against A.SW was produced by giving A mice 5 challenges with A.SW tumors at intervals of 10 days; the antiserum was taken 5 days after the last challenge.

†Selected for growth in A.

‡Selected for growth in A.SW.

TABLE 15.—Serologic tests of 2 lines from LNSA, IVA and IVS, propagated in (A × A.SW)F_1 mice, and treated before each serial transplantation with A-anti-A.SW and A.SW-anti-A serum, respectively, in the presence of complement

	Effect of antiserum diluted 1:5					
	Line IVA			Line IVS		
Passage	Percent of unstained cells		Cytotoxic index	Percent of unstained cells		Cytotoxic index
	Control	Antiserum		Control	Antiserum	
1	90	15	0.83	90	10	0.89
2	76	12	0.84	71	21	0.70
3	68	49	0.28	72	15	0.79
4	72	7	0.90	72	6	0.92
5	54	7	0.87	54	9	0.83

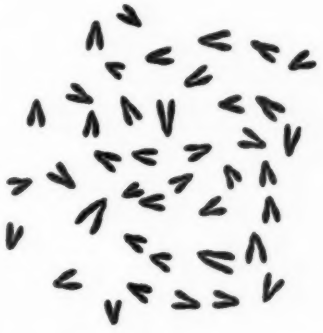
TABLE 16.—Distribution of cells with different chromosome numbers in LNSA, LNSB, and their variant sublines LNSAA, LNSAS, and LNSBS

Tumor			Number of chromosomes							Number of cells counted
Name	Passage	Last host	38	39	40	41	42	43	44	
LNSA	6	(A × A.SW)F_1			4	21	2	1	2	30
LNSA	6*	(A × A.SW)F_1			3	12				15
LNSAA	2†	A	2	1	7	14	2	3	1	30
LNSAA	4†	A preimmunized against A.SW			8	18	2	1	1	30
LNSAS	3‡	A.SW preimmunized against A	1	2	3	21	1	2		30
LNSAS	4‡	A.SW preimmunized against A			8	17	5			30
LNSB	4	(A × A.SW)F_1			9	21	2	1	1	34
LNSBS	1†	A.SW preimmunized against A		2	6	18	4			30
LNSBS	1†	A.SW preimmunized against A			2	24	4			30

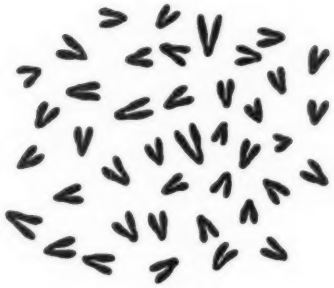
*Mesenteric lymph-node cells were studied.

†Number of passages in the parental host of selection; original tumor was serially transplanted for 3 transfer generations in (A × A.SW)F_1 before isolation of the variant.

‡Number of passages in the parental host of selection; original tumor was serially transplanted for 2 transfer generations in (A × A.SW)F_1 before isolation of the variant.



TEXT-FIGURE 5A.—Metaphase of LNSB. The tumor was taken from an A/Sn × A.SW mouse from passage 4. × 1,150



TEXT-FIGURE 5B.—Metaphase of LNSBS, a variant subline of LNSB, selected for growth in A.SW. The tumor was taken from an A.SW mouse preimmunized against A/Sn and represents the second passage of LNSBS. × 1,150

DISCUSSION

Characteristics of the Isoantigenic Variants

Six homozygous lymphomas serially transplanted to various host genotypes grew exclusively in genetically compatible mice. Sublines selectively compatible with one or the other of the parental strains were isolated from heterozygous F_1 lymphomas. Three such lymphomas originated in crosses between 2 IR mouse lines with a common strain A background but differing at the *H-2* locus. Two others were derived from F_1 crosses between unrelated strains. Similar findings have been reported by Klein and Klein, who studied carcinomas and sarcomas (*4*, *7–10*), and by Mitchison using lymphomas (*5*, *11*). The parental-strain sublines were called "variants" if they could breed true in their parental strains and grew in preimmunized mice, either immediately or after some passages in nonimmunized mice. The frequency of variant formation varied among different tumors.

Serologic demonstration of the isoantigens involved was performed with the cytotoxic technique of Gorer and O'Gorman (*14*, *15*). An advantage of this technique is that suspended cells can be directly examined for given isoantigens; the source of error introduced by contaminating stroma cells of the host is therefore of small or no importance in contrast to the procedure used with cells less sensitive to isoantibodies (*15*), in which the immunizing ability of the tissue as a whole is being determined by, *e.g.*, hemagglutination. The cytotoxic technique also permits a quantitative distinction between the isoantigen content of homozygous and heterozygous cells, as shown in the present paper. As yet only *H-2* isoantigens have been demonstrated by this method (*15*).

In contrast to cells of the original F_1 lymphomas sensitive to *H-2* isoantibodies directed against both parental strains, variant sublines capable of growing in preimmunized mice of one parental strain appeared to have lost their sensitivity to isoantibodies directed against the opposite

parent. This loss was permanent and could not be reversed by returning the variants to the original F_1 genotype or, in the LNSA lymphoma which was capable of nonspecific growth in nonimmunized mice of both parental strains, by carrying them in the opposite parental strain for as many as 12 serial passages. This agrees with the findings of Klein *et al.* (*10*) regarding the stability of variants isolated from methylcholanthrene-induced sarcomas of F_1 mice.

The variants were clearly different from nonspecific tumors, which grow indiscriminately across histocompatibility barriers.

Variant sublines with specific compatibility:	*Nonspecific tumors and sublines:*
Can only be derived in parental strains from heterozygous F_1 tumors.	Can be derived in foreign genotypes from both homozygous and heterozygous F_1 tumors.
Are specifically compatible with the same parental strain on further serial passage, to the exclusion of other genotypes.	Are not specifically compatible with the same strain on further serial passage but retain nonspecific behavior in other genotypes.
Grow in mice of the parental strain even if they are preimmunized against the *H-2* isoantigens of the other parent.	Growth can usually be prevented in preimmunized mice.
Characterized by irreversible loss of the *H-2* isoantigens specifically derived from the opposite parental strain, occurring in conjunction with the development of the ability to grow in preimmunized mice.	No *H-2* isoantigenic losses can be demonstrated by serologic technique.

Such nonspecific tumors occur both among homozygous and heterozygous sarcomas and carcinomas, but they are more infrequent in lymphomas. Specifically, compatible variants were only found in heterozygous F_1 tumors and were only compatible with a parental strain. Nonspecific tumors do not breed true in a particular genotype to the exclusion of other strains, while variants grow selectively in their parental strain of selection and do not revert to the original state. In contrast to specific variants, the growth of nonspecific tumors in foreign genotypes can usually be prevented by the use of preimmunized hosts. Another way of differentiating between the two phenomena is by the cytotoxic technique. In contrast to true variants, nonspecific takes inhibited by preimmunization, such as those obtained from, *e.g.*, the LNSA lymphoma in DBA/2 mice, did not show isoantigenic losses and were as sensitive to *H-2* isoantibodies as the original lymphoma was. True variants isolated from the same tumor by selective transfer in the appropriate parental host were characterized by stable and irreversible antigenic losses.

Both (A $\times$ A.SW)F_1 lymphomas studied, LNSA and LNSB, gave more A.SW than A variants, as in the findings of Klein and Klein on other tumor types (*7*). In contrast to the A.SW variants, which were immediately compatible with preimmunized mice after their first isolation, both A

strain variants studied, LNSAA and LNSBA, required a certain number of serial transfers in nonimmunized A mice before they grew in preimmunized animals. The difference between A and A.SW cannot be explained satisfactorily at present, but it may be assumed to be due to a difference in the strength of the *H-2* barriers separating the 2 strains. Cytotoxic tests on F_1 lymphomas failed to reveal any difference in their sensitivity to the reciprocal *H-2* isoantibodies directed against A or A.SW, respectively, provided the antiserums had been prepared similarly. Non-*H-2* differences between A and A.SW have been shown to exist by skin grafting by Linder and Klein (*16*). This cannot explain the difference in variant formation between the 2 strains for 2 reasons. As mentioned previously, the serologic tests showed that *H-2* isoantigens of both parents were present in the F_1 cells propagated in a parental strain as long as the tumor remained incompatible with preimmunized mice. This means that *H-2* differences rather than non-*H-2* factors were delaying variant formation. Furthermore, in spite of the more pronounced non-*H-2* differences that must exist between 2 unrelated strains such as DBA/2 and C3H as compared to 2 IR lines with a common genetic background, it was actually easier to isolate variants in both parental strains from (DBA/2 $\times$ C3H)F_1 lymphomas than from (A $\times$ A.SW)F_1 tumors.

The Mechanism of Variant Formation

The irreversible character of the isoantigenic losses characterizing the variants, together with the fact that they could be obtained from heterozygous but not from homozygous tumors, suggests a genetic change. However stable changes of cellular phenotype have been described that were not due to changes at the genetic level (*21*). Such changes, depending on "mechanisms that regulate the expression of genetic potentialities" rather than on "truly genetic mechanisms that regulate the maintenance of the structural information" (*22*), have been called "epigenetic" by Nanney (*21*). It is impossible to distinguish clearly between genetic and epigenetic mechanisms in cells that cannot be hybridized, and all discussions must be based on circumstantial evidence. As a rule epigenetic changes are less stable and can often be induced in a predictable way (*21*).

From available evidence it cannot be decided whether the mechanism of variant formation is genetic or epigenetic. If genetic, the variant cells would be formed randomly and spontaneously in the F_1 genotype of origin or during the period of temporary background growth in the parental host in which they would be selected by the homograft reaction. Isoantigenic variants that possibly were of such a nature have been found pre-existing among erythrocytes from normal human blood of the AB type (*23*). Since the progeny of the variant cells could not be obtained in this system, it cannot be excluded, however, that the variants represent some physiologic state of red cell development rather than a permanent and heritable cellular change.

Some findings suggest that immunoselection of the type considered may occur during variant formation. Examination of the detailed chro-

mosome complement of variants derived from a methylcholanthrene-induced (A × A.SW)F_1 sarcoma by Bayreuther and Klein (*12*) showed that 2 variants obtained in 2 different A.SW mice inoculated with aliquots from the same pool of cells had the same stem line. In contrast, a number of other A.SW variants obtained at different times from the same tumor were characterized by differences in their chromosome complement. The identity of the 2 variants obtained from the same pool can be best explained by assuming the pre-existence of a variant clone in the original F_1 tumor. In agreement with this assumption, it has been found, both by Klein and Klein (*8*) and in this study, that variants can sometimes be obtained directly by inoculating the F_1 tumor into preimmunized parental mice, a procedure that reduces the period of background growth in the selective parental host to a minimum and would therefore mostly concentrate preformed variants. When the yield of variants was studied by titrating various cell doses of the F_1 tumor in the parental strain, no good correlation was found, however, between the size of the inoculated population and the number of variants obtained. This would indicate that variant formation is usually not due exclusively to the selection of preformed clones from the F_1 tumor but to events taking place after inoculation into the parental host.

Among the various possible genetic mechanisms that may lead to a loss or decrease of *H-2* isoantigens, several may be considered on the basis of available data. The findings of Bayreuther and Klein (*12*) suggest a chromosomal change, possibly involving the chromosome segment carrying *H-2*, or modifying the expression of the *H-2* carrying chromosome more indirectly by a change in genetic balance. In their methylcholanthrene-induced sarcoma cells, chromosomal differences could be found separating maternal-strain variants isolated at different times from the sarcoma of origin. In our lymphomas, however, no differences were seen between the F_1 tumors and their variants when the cytologic technique was used, although minor alterations such as inversions and translocations might have remained undetected. The contradictory results may be due to the greater chromosomal variability in methylcholanthrene-induced sarcomas, already obvious in primary tumors (*13*). The chromosomal differences between the different sarcoma variants may merely reflect clonal variation in the tumor without being causally correlated to variant formation.

Other genetic changes that may be considered include point mutations, transfer of genetic material between tumor cells or between tumor and host cells, *e.g.*, recombination or transduction, or mitotic crossing over.

In point mutation, a loss mutation would have to be postulated, since specifically growing variants have never been found in other genotypes than those corresponding to the parental IR strains entering the original F_1 cross. Loss mutations are made less likely, however, by the finding that X irradiation does not influence variant formation to any degree (*10*).

There is no evidence to indicate that transfer of genetic material between cells is responsible for variant formation. Selection of variants

from a certain F_1 tumor in another F_1 genotype rather than in a parental strain are relevant in this connection. The selective F_1 host in question has been produced by crossing one of the parental strains of the tumor with a third, unrelated strain. If variant formation would be due to the "transduction" of genetic material from host to tumor cells, one may expect to recover some of the *H-2* isoantigens of the third strain from some of the variants. This never occurred, however, neither in the present study nor in the transplantation tests of Klein, Klein, and Hellström (*10*). Experiments designed to demonstrate the occurrence of possible genetic recombination between tumor cells have also given negative results (*10*).

Mitotic crossing over is a possible explanation. It would lead to homozygous rather than hemizygous variants, in the *H-2* genotype of the parental strain in which they have been selected. This expectation is fulfilled by the finding that the variants were more like the homozygous lymphomas of the parental strain in which they have been selected than the original F_1 lymphoma from which they have been derived, in their sensitivity to cytotoxic antibodies. They also grew more specifically, being less liable to take in foreign strains than the original F_1 tumor, similar to homozygous tumors (*10*). It must be emphasized, however, that phenotypic similarity to a homozygous cell is by no means convincing evidence of genetic homozygote. A genetically hemizygous cell may phenotypically appear like a homozygote, if the products of the remaining allele replace structurally the products of the missing allele. Another reason why somatic crossing over cannot be accepted as an interpretation with confidence lies in the highly unequal frequency of the variants, obtained in the 2 parental strains, from tumor to tumor. However this may be due to the previously discussed unequality of the barriers separating the F_1 from the parental strains. Somatic crossing over has been demonstrated in *Drosophila* (*24*) but not in mammalian cells.

Although immunoselection of spontaneously formed isoantigenic variants from the F_1 population probably explains some variant formation, as discussed previously, it has not been possible to prove its occurrence by selection of variant cells after antiserum treatment *in vitro*. No cells resistant to *H-2* isoantibody could be selected by 4 consecutive treatments *in vitro* of the (A $\times$ A.SW)F_1 lymphoma LNSA with A-anti-A.SW and A.SW-anti-A serum, respectively. Such a failure may be due to either the absence of preformed variant cells or any such variants may not yet have undergone all the modifications necessary to lead to a fully parental-compatible, *i.e.*, antibody resistant, variant cell. More specifically, since *H-2* is a complex locus, and since several *H-2* isoantigens probably determined by different sites on the chromosome (*25*) must be lost simultaneously to give rise to a variant compatible with one parent and resistant to isoantibody, preformed variants would have to lose all antigens in question to be detected by the *in vitro* technique used. In fact it has been found that variant formation proceeds

in several steps involving sequential losses of *H-2* isoantigens in methylcholanthrene-induced sarcomas (*8*, *10*).

The period of background growth seems to be of great importance for variant formation since passage via nonimmunized mice or newborns may facilitate the selection of variants capable of growing in preimmunized mice. In the present study, passage via newborns increased the yield of variants that could also develop spontaneously, similar to the findings of Mitchison (*11*), though it was not possible by this procedure to isolate variants that could not be obtained in adult mice. The significance of background growth is not clear; in the case of a genetic change it may act by permitting additional genetic variation to occur and allowing additional adjustments in incomplete variants. Alternatively, an antiserum-mediated epigenetic mechanism would obviously be facilitated by a more prolonged exposure to the active principle. The latter phenomenon would possibly be analogous to certain antiserum-induced serotype transformations described in *Paramecium aurelia* (*26*). The transformed serotypes usually revert to the original state after some generations, however. The antibodies determine which serotype-determining genes are expressed. In heterozygous *Paramecia*, the expression of one of the two genes determining the serotype at a certain moment is often suppressed, but generally can be restored by modifications of the environment (*27*). Variant formation appears to be different from these phenomena, however, since the missing isoantigens were permanently lost and could not be restored to the variant cells.

Several examples of induced changes in the transplantation specificity of tumor cells have been reported; the best analyzed cases are immunological enhancement, as described by Kaliss (*28*), and the Barrett-Deringer effect (*29*, *30*).

In this investigation, some phenomena were observed that appear to be analogous with enhancement. Thus the A.SW variant LNSAS could grow in A.BY mice for 2 transfer generations, following 1 passage in newborn A.BY mice. Subsequently, however, it could not be propagated further in A.BY. Similarly, one LNSB subline, derived from newborn (A.SW $\times$ DBA/2)F_1 mice, grew in all nonimmunized A.SW $\times$ DBA/2 mice during the first transfers, but died out after some generations. In both cases, serologic tests did not reveal any loss of the *H-2* isoantigens. It is possible that those observations, in particular, and perhaps the growth of nonspecific tumors, in general, is due to immunologic enhancement.

Since all cases of enhancement hitherto described have concerned neoplasms of homozygous origin, mostly sarcomas and carcinomas, it is conceivable that more stable changes could be obtained by enhancement of heterozygous leukemia cells in one of their parental strains. For other reasons, however, it is rather unlikely that variant formation is analogous to immunological enhancement, as described by Kaliss (*28*). The permanent loss of detectable *H-2* isoantigens from the variant and their ability to grow in parental mice preimmunized 7 days prior to grafting

distinguish them from enhanced tumors. It is also puzzling why enhancement should give rise to a higher number of variants in one parental strain, *e.g.*, more takes in A.SW than in A, since there is no difference in the sensitivity of the cells to *H-2* isoantibodies directed against A or A.SW *in vitro*.

The Barrett-Deringer effect is similar to the formation of isoantigenic variants, since it represents a permanent decrease in the histocompatibility requirements of tumors: A homozygous mammary carcinoma permanently acquires, after transplantation to F_1 mice from a cross between the strain of origin and a resistant strain, an ability to grow more readily in F_2 or backcross mice from the same outcross than in the original line (*29*, *30*). Klein and Klein (*31*) have shown that the change could not be due to selection of preformed variants or to genetic interaction with host cells. This, together with the fact that the difference disappears in preimmunized mice, would classify it as an epigenetic phenomenon (*32*). However, the Barrett-Deringer effect probably does not concern *H-2* (*7*).

Although it has been thus established that the isoantigenic variants isolated from F_1 tumors in one or the other of the parental strains have irreversibly lost the *H-2* isoantigens derived from the opposite parental strain, it is still unknown whether the mechanism of the variant formation is at the genetic or epigenetic level. Further investigations are in progress to clarify this problem, involving attempts to induce variant formation by mutagenic treatment, continued trials to select *H-2* isoantibody-resistant cells *in vitro*, and studies of the effect of immunological enhancement on the formation of variants.

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Further Studies on Isoantigenic Variation in Mouse Carcinomas and Sarcomas [1,2]

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SUMMARY

Further studies have been made on the occurrence and nature of isoantigenic variant formation in sarcomas, carcinomas, and lymphomas arising in F_1 hybrid mice derived from the outcross of 2 isogenic-resistant (IR) lines, which have confirmed the individuality of different tumors of the same type. No variants have been obtained in a small number of estrogen-induced and dependent testicular tumors. Variants compatible with a parental strain could also be isolated from tumors arising in F_1 hybrids derived from crossing 2 unrelated inbred strains of mice.—J. Nat. Cancer Inst. 25: 271–294, 1960.

PREVIOUS PAPERS (*1–6*) have described tumors, originating in F_1 hybrids from 2 isogenic-resistant (IR) lines of mice (*7*), that have been used for studying isoantigenic variation in populations of sarcoma and carcinoma cells, and Hellström (*8*) extends the system to lymphomas, which have the additional advantage of direct sensitivity to cytotoxic isoantibodies (*9*, *10*). The motivation for this work, the experimental system used, and the earlier results have been described in detail in the previous papers (*1–6*) and in articles by Lederberg (*11*) and Mitchison (*12*, *13*). The present paper brings the results on F_1 sarcomas and carcinomas up to date, presents new data on tumors originating in homozygotes and in F_1 hybrids derived from the outcross of 2 unrelated strains, describes experiments intended to clarify the characteristics of the variants, as compared to the original tumors, and tries to elucidate, as far as possible, the nature of variant formation.

MATERIALS AND METHODS

Our materials and methods have been described in detail in a previous publication (*3*) and in a paper by Hellström (*8*); any deviation from the procedure previously used will be described in connection with the results.

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[3] The skillful and dependable technical assistance of Miss Barbro Lindström, Miss Maj-Lis Eriksson, and Miss Birgitta Pettersson is gratefully acknowledged.

RESULTS

Further Data on Variant Formation in F_1 Tumors Originating in F_1 Hybrids From 2 IR Lines

Detailed results on these tumors have been given in previous publications (*1–6*), and an up-to-date summary is given here (table 1). The previous impression that the maternal strain is regularly preferred in variant formation could not be verified when tumors induced in reciprocal F_1 crosses became available. The previously described individuality of tumors with regard to variant formation was fully verified and can now be extended to mammary carcinomas and also, as shown by Hellström (*8*), to lymphomas. Table 1 gives only a schematic illustration of this, but it may be pointed out that the quantitative and qualitative behavior of a given tumor line with regard to variant formation was an individual and quite stable characteristic of a given line, even if it did

TABLE 1.—Summary of data on variant formation in tumors originating in F_1 hybrids from 2 IR strains

Genotype P_1 P_2	Tumor type	Number of tumor lines tested*	Number of tumor lines giving no variants†	Number of tumor lines giving variants in P_1 exclusively	Number of tumor lines giving variants in P_2 exclusively	Number of tumor lines giving variants in both parents	Non-specific
(A.CA ♀ × A.BY ♂)F_1	MC-sc.‡	8	3	0	4	1	0
(A.CA ♂ × A.BY ♀)F_1	MC-sc.	6	4	0	1	0	0
(A.BY ♀ × A.CA ♂)F_1	Mammary carcinoma§	7	5	0	1	1	0
(A.BY ♀ × A.CA ♂)F_1	Lymphoma‖	1	0	0	1	0	0
(A.SW ♂ × A.CA ♀)F_1	MC-sc.	4	1	0	1	1	1
(A.SW ♀ × A.CA ♂)F_1	MC-sc.	6	2	2	1	1	0
(A.CA ♂ × A ♀)F_1	MC-sc.	6	1	2	0	3	0
(A.CA ♀ × A ♂)F_1	MC-sc.	6	3	2	0	1	0
(A.CA ♀ × A ♂)F_1	Mammary carcinoma§	3	1	0	1	1	0
(A ♂ × A.SW ♀)F_1	MC-sc.	14	4	2	4	0	4
(A ♀ × A.SW ♂)F_1	MC-sc.	9	3	0	4	1	1
(A ♂ × A.SW ♀)F_1	Mammary carcinoma§	1	0	0	1	0	0
(A ♀ × A.SW ♂)F_1	Mammary carcinoma§	1	1	0	0	0	0
(A ♂ × A.SW ♀)F_1	Lymphoma‖	2	0	0	0	2	0
(A × A.BY)F_1	MC-sc.	3	2	0	0	1	0
(A.BY × A.SW)F_1	MC-sc.	3	0	0	1	0	2

*Each tumor was tested in both parental strains during its first 5 transfer generations; most tumors were carried and tested through 10 transfers. As soon as takes were obtained in one parental strain, consistent efforts were made to establish variant lines from them.

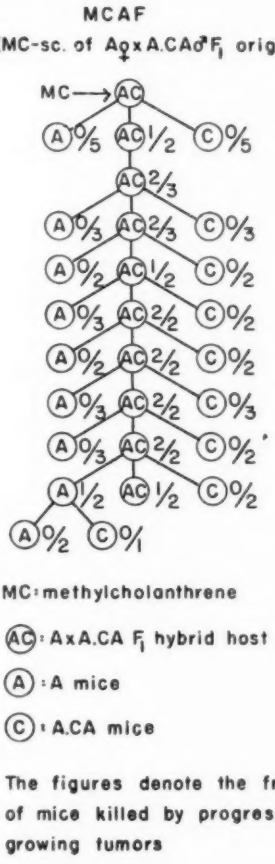
†Either there were no takes in any parental mice at all, or they failed to breed true in the same genotype on repeated testing.

‡Methylcholanthrene-induced sarcoma.

§Spontaneous or estrogen-induced.

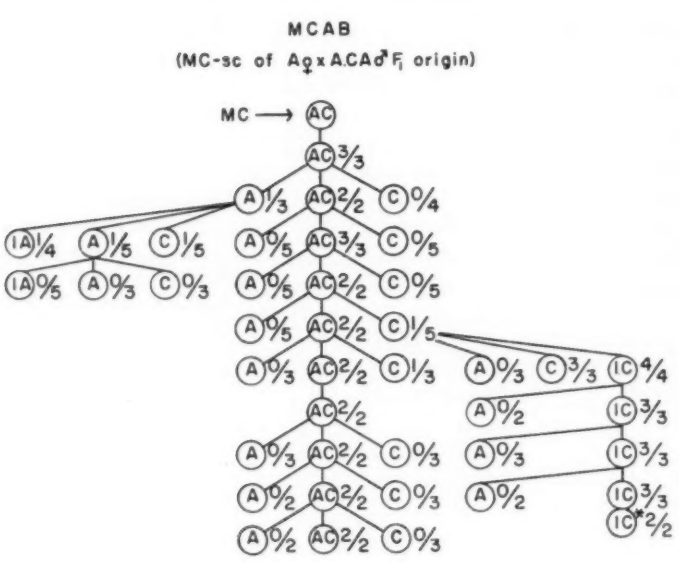
‖Spontaneous or estrogen-induced.

sometimes undergo random and unpredictable changes. Text-figures 1, 2, and 3 illustrate the transplantation history of 3 sarcomas, all induced by the same dose of methylcholanthrene in the subcutaneous tissue of (A♀ × A.CA♂)F_1 mice. They all grew at approximately the same rate and showed a histologically similar picture of undifferentiated spindle-cell sarcoma. Tumor MCAF (text-fig. 1) showed no tendency toward variant formation; a single take in a parental A strain mouse did not grow in A mice on repeated testing and was therefore classified as a "false positive." Tumor MCAB (text-fig. 2) gave an A.CA variant that was immediately compatible with preimmunized A.CA mice but refused to grow in A mice. An occasional take in an A mouse turned out to be a false positive. Sarcoma MCAD (text-fig. 3) behaved quite differently; it gave rise to a considerable number of takes in both parental strains. The takes that were tested turned out to be true variants. The history



TEXT-FIGURE 1.—Transplantation history of a tumor giving no variants.

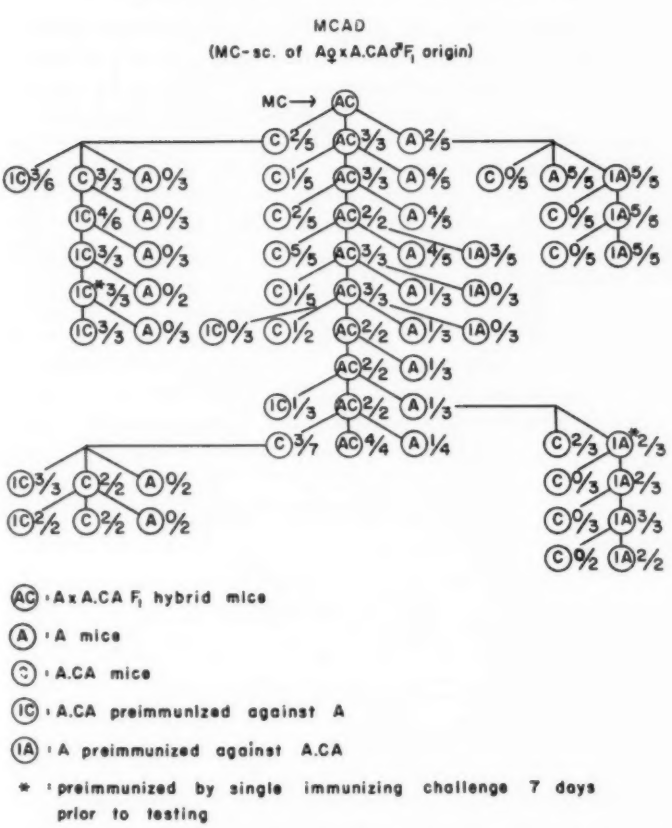
553742—60——6


MCAB
(MC-sc of A♀xA.CA♂ F1 origin)
MC → AC
AC 3/3
A 1/3 AC 2/2 C 0/4
IA 1/4 A 1/5 C 1/5 A 0/5 AC 3/3 C 0/5
IA 0/5 A 0/3 C 0/3 A 0/5 AC 2/2 C 0/5
A 0/5 AC 2/2 C 1/5
A 0/3 AC 2/2 C 1/3 A 0/3 C 3/3 IC 4/4
AC 2/2 A 0/2 IC 3/3
A 0/3 AC 2/2 C 0/3 A 0/3 IC 3/3
A 0/2 AC 2/2 C 0/3 A 0/2 IC 3/3
A 0/2 AC 2/2 C 0/3 IC* 2/2
AC : AxA.CA F1 hybrid host
A : A mice
C : A.CA mice
IC : A.CA preimmunized against A
IA : A preimmunized against A.CA
* : preimmunized by single immunizing challenge 7 days prior to testing

TEXT-FIGURE 2.—Transplantation history of a tumor giving rise to variants in one parental strain.

of this tumor shows some other interesting points that will be treated in more detail in later sections. While the original tumor line regularly gave a number of takes in both parental strains, each variant failed consistently to grow in the opposite parental strain. Variants that were compatible with mice of one parental strain, preimmunized against the isoantigens of the opposite parent in the ordinary way, *i.e.*, by giving a single subcutaneous challenge of a tumor containing the required isoantigens, approximately 1 month prior to testing, also grew if a single immunizing challenge of mixed liver, kidney, and spleen tissue was given intraperitoneally 7 days prior to inoculation. This schedule is most likely to exclude immunological enhancement (*14*). Finally, text-figure 3 shows that in some instances it was possible to obtain a small number of takes in preimmunized parental mice directly from the original line of the tumor.

Preliminary results are available on still another tumor type which has been induced by implanting estrogen pellets into the interstitial cells of


MCAD
(MC-sc. of A♀xA.CA♂F1 origin)
MC→
AC = A x A.CA F1 hybrid mice
A = A mice
CA = A.CA mice
IC = A.CA preimmunized against A
IA = A preimmunized against A.CA
* = preimmunized by single immunizing challenge 7 days prior to testing

TEXT-FIGURE 3.—Transplantation history of a tumor giving rise to variants in both parental strains.

the testis of (A♂ × A.SW♀)F_1 males, by the procedure of Andervont *et al.* (*15*). The tumors, dependent on estrogenic stimulation, were incapable of growing in untreated males. In untreated females the tumors did not grow or grew much slower than in estrogen-treated females. Although the number of animals used is still very small, the data are shown in table 2 since they have a certain special interest. Up to the present, the 2 tumors tested have not given rise to variants in the parental strains. It remains to be seen whether this behavior can be verified in a larger number of mice and, if so, whether it is peculiar to these tumors in particular or to hormone-dependent tumors in general.

Tumors Originating in F_1 Hybrids from 2 Unrelated Strains

IR lines have been shown to differ by more than one histocompatibility gene (*7*, *16*). This may explain why parental variants, selected from

tumors originating in F_1 hybrids of 2 IR lines, sometimes undergo more than one demonstrable change before they become fully compatible with their new host (*6*). In this connection, it appeared to be of interest to investigate the behavior of tumors originating in the F_1 hybrid progeny of 2 entirely unrelated strains that differ with regard to a much greater number of histocompatibility factors than the 2 IR lines do. Three sarcomas were induced by methylcholanthrene in (DBA/2 × C57BL)F_1 hybrids. They were tested for variant formation in the usual way. The results are shown in table 3. It appears that M5DD took frequently in C57BL mice and in (A × C57BL)F_1 hybrids. Further tests indicated, however, that the tumors appearing in C57BL were not fully compatible with their new hosts, since they did not grow regularly in preimmunized C57BL mice and died out after a few passages. One take in an (A × C57BL)F_1 mouse (M5DDA7) could be propagated in (A × C57BL)F_1 mice through 4 consecutive passages, and may eventually turn out to be a true variant, though more extensive tests, including preimmunized

TABLE 2.—Transplantation tests with estrogen-induced and dependent testicular tumors originating in (A ♂ × A.SW ♀)F_1 mice

Designation	Number of serial transfers	Host*							
		Bearing estrogen pellet						Without pellet	
		(A × A.SW)F_1		A		A.SW		(A × A.SW)F_1	
		♂	♀	♂	♀	♂	♀	♂	♀
TNSB	4	9/16	8/18	—	0/14	—	0/11	0/17	8/21
TNSC	5	8/11	12/15	0/5	0/2	0/5	0/1	0/14	7/15
TNSD	2	3/6	0/5					0/6	0/7
TNSE	1	2/2	0/3					0/4	0/4

*Fractions denote the number of mice killed by progressively growing tumors over total number inoculated.

mice, will be needed to prove this point. Since the A strain is known to contain the same *H-2*-determined antigenic components as DBA/2, in addition to other *H-2* components (*17*), the homograft barrier separating a (DBA × C57BL)F_1 hybrid tissue, the original tumor genotype, from an (A × C57BL)F_1 recipient is essentially due to other than *H-2* differences. Since *H-2* is considered a very strong barrier against homotransplantation, such a non-*H-2* barrier can be expected to be more easily transgressible than the *H-2* difference which separates the hybrid from its C57BL parent.

With the M5DE sarcoma, only 1 take was obtained in the C57BL strain; this did not breed true on further testing. The third tumor, M5DF, gave 1 take in a DBA/2 mouse in which the other 2 tumors did not grow. On further testing, this tumor, denoted M5DFD, appeared to be highly compatible with DBA/2 mice, in which it grew regularly after 5 passages, including hosts preimmunized against C57BL tissue. Another apparently true variant of this tumor was obtained in (A × C57BL)F_1 hybrids, in which it has been carried for 8 consecutive passages until the present. Some takes were obtained with this variant in the parental

Table 3.—Transplantation tests with 3 sarcomas induced in (DBA/2 × C57BL)F_1 hybrids*

Table 3.—Transplantation tests with 3 sarcomas induced in (DBA/2 × C57BL) F_1 hybrids*

Tumor	(DBA/2 × C57BL) F_1	DBA/2		C57BL		(A × C57BL) F_1	(C3H × C57BL) F_1	(DBA/2 × A) F_1	(DBA/2 × C3H) F_1	C57 leaden	Note
		Untreated	Preimmunized against C57BL	Untreated	Preimmunized against DBA/2						
M5DD	25/26	0/28		25/36 (*a*, *b*)	0/8	5/6 (*c*)		0/3	0/3	0/2	Twelve passages.
(*a*) M5DD5-1 taken from C57BL mouse inoculated with M5DD.		0/3		1/5	0/3				0/3		Grows for one passage in C57BL mouse, dies out in second. False positive.
(*b*) M5DD5-2 taken from C57BL mouse inoculated with M5DD.		0/11		9/12	1/7						Grows for 3 serial passages in C57BL, dies out in the fourth. False positive.
(*c*) M5DDA7, taken from (A × C57BL) F_1 mouse inoculated with M5DD.		0/4		4/4	0/5	8/8	2/6		0/5		Four passages in (A × C57BL) F_1. Appears to be true variant.
M5DE	17/18	0/17		1/27 (*d*)		0/2		0/4			Nine passages.
(*d*) M5DE5 taken from C57BL mouse inoculated with M5DE.		0/3		0/3	0/3					(A × ABY) F_1 0/3	False positive.
M5DF	21/21	1/17 (*e*)		0/30		2/4 (*f*)				(A × ABY) F_1 0/2	Ten passages.
(*e*) M5DFD taken from DBA/2 mouse inoculated with M5DF.		12/14	9/10	2/14	0/5	0/4					Seven passages in DBA/2. Appears to be true variant.
(*f*) M5DFA5 taken from (A × C57BL) F_1 mouse inoculated with M5DF.		1/13 (*g*)		2/10 (*h*)		19/19	2/5		0/2		Eight passages in (A × C57BL) F_1. Appears to be true variant.
(*g*) M5DFA5-D taken from DBA/2 mouse inoculated with M5DFA5.		5/7	3/5	0/4		2/3	0/2				Grows for 2 passages in DBA/2, dies out in third. False positive.
(*h*) M5DFA5-56 taken from C57BL mice inoculated with M5DFA5 (2 tumors tested).		0/1		0/4	0/4				0/2		False positives.

* Fractions denote the number of mice killed by progressively growing tumors over total number inoculated in recipient strain mentioned.

DBA and C57BL strains, respectively. These did not turn out to be true variants on repeated testing.

Preliminary as they are, these experiments show that it is possible to establish true variant lines even from tumors induced in F_1 hybrids derived from the outcross of 2 unrelated strains.

Tumors Originating in Homozygous Mice

Table 4 summarizes the tests made with tumors originating in various homozygous IR genotypes and tested for their possible ability to give rise to variants compatible with another homozygous IR strain or with F_1 hybrids derived from the crossing of 2 other IR lines. Occasional takes were obtained in such genotypes. On further testing, however, these always turned out to be false positives, *i.e.*, they failed to grow in the same genotype or were nonspecific forms that grew indiscriminately in all kinds of mice. They were in no case specific for the particular foreign genotype from which they were isolated. This is in sharp contrast to the findings with heterozygous tumors.

Variants Obtained From F_1 Tumors in Semi-isologous F_1 Hosts

Since the 4 IR lines contained 4 different alleles at the *H-2* locus, it was possible to study whether or not a tumor originating in a certain F_1 hybrid (*e.g.*, *H-2^{a}H-2^s*) can give rise to variants specifically compatible with a semi-isologous F_1 genotype produced by outcrossing one of the parental strains with a third strain. Table 5 exemplifies the results obtained in 7 tests of this type; a number of other experiments gave essentially the same result. Whenever takes obtained in a semi-isologous F_1 were tested further and turned out to be specific variants, they were not specific for the F_1 in question but for the parental strain entering the outcross.

Further Studies on the Characteristics of the Variants

Specificity.—True variants were often more specific in their transplantation characteristics than the original F_1 tumor from which they had been derived. This was particularly apparent with tumors giving rise to variants in both parental strains. While the original tumor took in both parental types with a certain frequency, a variant derived in and compatible with P_1 failed to grow in P_2. A number of such cases is illustrated in table 6.

Irreversibility.—The previously described (*5*, *6*) permanence and irreversibility of variants after returning them to the original F_1 genotype was confirmed in more extensive tests involving more prolonged F_1 passage. The variants maintained their characteristics in their ability to grow in untreated or in preimmunized parental-strain mice, respectively. Some representative cases are illustrated in table 7.

Attempts to "switch" variants experimentally.—Several attempts were made to change the specificity of the variants from one parental type to

TABLE 4.—Summary of transplantation tests on tumors originating in homozygous IR mice

Genotype of origin	Tumor type	Number of tumors tested†	Recipient genotype*									
			A	A.SW	A.CA	A.BY	(A.CA × A.SW)F_1	(A.BY × A.SW)F_1	(C3H × A.SW)F_1	(DBA/2 × A.SW)F_1	(A.CA × A.BY)F_1	(A. × A.BY)F_1
A	MC-sc.	5	78/82	3/53 (0/8)	1/40 (2/5)‡	1/56 (2/6)‡	0/13	0/2	0/10	1/15		
A	Lymphoma§	1	21/23	0/29	0/4	0/6					0/2	
A	Mammary carcinoma	1	10/10	0/5	0/2	0/6						
A.SW	MC-sc.	3	0/72 (0/4)	88/92	1/58 (2/5)‖	1/64 (1/6)‖					1/9‖	0/12
A.SW	Lymphoma§	2	0/20	49/49	0/4	0/6	(A × A.SW) F_1 13/13				0/6	
A.SW	Mammary carcinoma	1	0/9	13/13	0/8	0/8					0/12	0/3
A.CA	MC-sc.	5	1/80‡ (0/5)	3/74‖ (0/3)	81/85	0/76 (1/5)‖	(A × A.SW) F_1 0/6	2/35‖				0/6
A.CA	Lymphoma§	1	0/11	0/9	19/19	0/19						
A.CA	Mammary carcinoma	1	0/9	0/5	2/3	0/4						

*Fractions denote the number of mice killed by progressively growing tumors over total number inoculated. The figures in parentheses refer to tests made on mice pretreated with tumor homogenate, as described in connection with the enhancement experiments.

†Most tumors have been tested during their first 10 transfer generations; a few were carried for a shorter period of time.

‡One tumor tested further: nonspecific.

§*Cf.* paper by Hellström (*8*).

‖One or 2 tumors tested further: false positive(s).

TABLE 5.—Examples of variants selected from heterozygous F_1 tumors in semi-isologous F_1 genotypes produced by outcrossing one parental strain with a third IR line

Name	Genotype of origin	Type	F_1 hybrid from which variant was selected*	Further testing of variant*: Variant fully or partially compatible with genotypes	Further testing of variant*: Variant incompatible with genotypes	Conclusion
BNSB	(A♂ × A.SW♀)F_1	Mammary carcinoma	(A.SW × A.CA)F_1 (3/3)	(A.SW × A.CA)F_1 untreated (2/2) and preimmunized against A (3/3); A.SW untreated (2/2) and preimmunized against A (2/2); (A.SW × A.BY)F_1 untreated (2/2) and preimmunized against A (2/2)	A, untreated (0/4) and preimmunized against A.SW (0/2); (A × A.BY)F_1 (0/3)	A.SW variant
MNSI	(A♂ × A.SW♀)F_1	MC-sc.	(A.SW × A.BY)F_1 (1/5)	(A.SW × A.BY)F_1 untreated (2/3) and preimmunized against A (1/4); A.SW untreated (10/10) and preimmunized against A (5/10); (A.SW × DBA/2)F_1 preimmunized against A (3/3)	Untreated A (0/5) and (A × A.CA)F_1 (0/5)	Partially compatible A.SW variant
MSCE	(A.CA♂ × A.SW♀)F_1	MC-sc.	(A.SW × A.BY)F_1 (1/3)	(A.SW × A.BY)F_1 untreated (2/2) and preimmunized against (2/2); A.SW untreated (2/2) and preimmunized against A.CA (6/6)	Untreated A.CA (0/4) and (A × A.CA)F_1 (0/2)	A.SW variant
MSCE	(A.CA♂ × A.SW♀)F_1	MC-sc.	(A × A.CA)F_1 (3/3)	(A × A.CA)F_1 preimmunized against A.SW (4/5); A.CA untreated (6/6) and preimmunized against A.SW (33/33); (A.CA × A.BY)F_1 preimmunized against A.SW (2/2)	Untreated A.SW (0/8) and (A × A.SW)F_1 (0/2)	A.CA variant

MSCK	(A.CA♂ × A.SW♀)F_1	MC-sc.	(A × A.CA)F_1 (3/3)	(A × A.CA)F_1 preimmunized against A.SW (5/5); A.CA untreated (4/4) and preimmunized against A.SW (4/5)	Untreated A.SW (0/4) and (A.BY × A.SW)F_1 (0/2)	A.CA variant
MNSL	(A♂ × A.SW♀)F_1	MC-sc.	(A × A.CA)F_1 (2/3)	(A × A.CA)F_1 untreated (2/2) and preimmunized against A.SW (4/5); A untreated (6/6) and preimmunized against A.SW (3/3); (A × A.BY)F_1 untreated (3/3) and preimmunized against A.SW (7/7)	Untreated A.SW (0/6) and (A.SW × A.BY)F_1 (0/8)	A variant
MACD	(A♂ × A.CA♀)F_1	MC-sc.	(A × A.SW)F_1 (1/5)	(A × A.SW)F_1 untreated (6/6) and preimmunized against A.CA (4/9); A untreated (9/11) and preimmunized against A.CA (8/13); (A × A.BY)F_1 untreated (2/2)	Untreated A.CA (0/5), A.SW (0/2), A.BY (0/3) (A.CA × A.BY)F_1 (0/3), (A.CA × A.SW)F_1 (0/3)	A variant

*Fractions denote the number of mice killed by progressively growing tumors over total number inoculated.

TABLE 6.—Specificity of variants compared to original F_1 tumor*

Tumor†	Genotype of origin‡ or of variant compatibility	Growth in P_1	Growth in P_2
MCAG	(A♀ × A.CA♂)F_1	8/24	16/28
MCAGA	A variant	18/18	0/6
MCAGC	A.CA variant	0/11	30/30
MCAD	(A♀ × A.CA♂)F_1	24/60	30/55
MCADA	A variant	29/31	2/24
MCADC	A.CA variant	0/13	20/26
MACC	(A♂ × A.CA♀)F_1	17/43	14/35
MACCA	A variant	21/22	1/9
MACCC	A.CA variant	0/13	19/19
MSAH	(A♀ × A.SW♂)F_1	7/18	4/19
MSAHA	A variant	13/15	0/12
MSAHS	A.SW variant	0/12	23/23

*The fractions denote the number of mice killed by progressively growing tumors over total number inoculated.

†Methylcholanthrene-induced sarcomas.

‡$P_1 \times P_2$.

the other. Since most P_1 variants failed to grow in adult P_2 animals under ordinary circumstances, the variants were inoculated into newborn mice of the P_2 type, or if adults were used, the grafts were removed before the onset of the homograft reaction. As a rule, these experiments gave negative results; the variants remained specific for P_1 and still failed to grow in P_2. One apparent exception was followed in some detail. This experiment was done with the MCAG sarcoma of (A♀ × A.CA♂)F_1 origin. Table 6 showed that specific variants could be established in both parental strains and table 7 illustrated the stability of one such variant which remained unchanged after 13 serial transfers in the F_1 type of origin. The actual experiment has been performed with this variant, MCAGA, highly compatible with the parental A strain, even if preimmunized against A.CA, and entirely incompatible with the A.CA parent (*cf.* table 7). In an attempt to "switch" the compatibility of MCAGA, it was inoculated into newborn A.CA mice in which it grew in 6 of 8 mice. Two of the resulting tumors were tested and the results are summarized in table 8. Both tumors grew in both A and A.CA mice, even if the recipients were preimmunized against the opposite parental type. They also grew in preimmunized A.BY and A.SW mice. The question arose whether or not the cells had lost *all H-2* isoantigens differentiating the A and A.CA strains. This was studied by determining the hemagglutinin titers against A and A.CA red cells, respectively, in A.CA and A mice treated with standard doses of heavily irradiated tumor cells. The tumor tissues were suspended in Ringer's solution in a 1:3 proportion, the suspensions were irradiated with 15,000 r, and 0.1 ml. was given subcutaneously per mouse and per challenge. Challenges were given at 10-day intervals, and the hemagglutinin titers were determined by the method of Gorer and Mikulska (*18*) after 3, 5, and 7 consecutive treatments. This procedure was previously found to be efficient when measuring the ability of nonspecific

TABLE 7.—Irreversibility of variants on prolonged passage in the F_1 genotype of origin

Tumor line (MC-sarcomas) or variant	Genotype of origin ($P_1 \times P_2$) or of variant compatibility	Growth in P_1	Growth in P_2	Number of serial return passages of established variants in F_1 type of origin	Growth in P_1	Growth in P_2
					Subsequent to passages in previous column	
MCAG	(A ♀ × A.CA ♂)F_1	8/24	16/28			
MCAGA	A variant	3/3 15/15*	0/6	13†	20/20 29/22*	0/22
MSCE	(A.SW ♀ × A.CA ♂)F_1	2/25	2/22			
MSCEC	A.CA variant	0/4	2/2 6/6‡	11§	0/6	4/4 29/29‡
MSWB	(A ♂ × A.SW ♀)F_1		(A.SW × C3H)F_1 19/50 0/15‖			(A.SW × C3H)F_1
MSWBSCI	(A.SW × C3H)F_1 variant (only untreated)		3/3 1/3‖	10¶		17/17 1/16‖
MSWBSCII	(A.SW × C3H)F_1 variant (including preimmunized)		3/3 3/3‖	11**		11/11 25/27**

*Preimmunized against P_2.
†Subsequent to 3 selective transfers in P_1.
‡Preimmunized against P_1.
§Subsequent to 3 selective passages in P_2.
‖(A.SW × C3H)F_1 hybrid preimmunized against A.
¶Subsequent to one selective passage in (A.SW × C3H)F_1.
**Subsequent to 2 selective passages in (A.SW × C3H)F_1.

TABLE 8.—Transplantation behavior and isoantigenicity of MCAGA (strain A variant of MCAG; *cf.* tables 6 and 7) after passage through newborn A.CA mice

		Transplantation behavior*						Isoantigenicity†	
		A		A.CA				Hemagglutinin titers	
Designation	History	Untreated	Preimmunized against A.CA	Untreated	Preimmunized against A	A.BY preimmunized against A	A.SW preimmunized against A	A.CA-anti-A	A-anti-A.CA
MCAGA	MCAG—variant in strain A, passaged in A or in (A × A.CA)F_1	23/23	44/44	0/28				512‡ 512§ 512‖	0‡ 0§ 0‖
Line 43-C	MCAGA—newborn A.CA—carried in A.CA for 13 passages		28/28	9/9	24/24	4/4	4/4	512‡ 512§ 512‖	0‡ 0§ 512‖
Line 43-C-A	Derived from line 43-C after 4 passages in A.CA; carried in A for 7 passages.		16/16	1/1	11/12			512‡ 512§ 512‖	0‡ 0§ 0‖
Line 43b-C	Established independently: MCAGA—newborn A.CA—carried in A.CA for 11 passages.		21/21	2/3	18/18	3/3	3/3	512‡ 512§	0‡ 256§

*Fractions denote the number of mice killed by progressively growing tumors over total number inoculated.

†Measured as the ability of heavily irradiated (15,000 r) inoculums to provoke hemagglutinin formation in A mice (anti-A.CA) and in A.CA mice (anti-A), respectively. The titers were determined after 3, 5, and 7 challenges given at 10-day intervals. Hemagglutination was measured 5 days after the last challenge.

‡After 3 challenges.

§After 5 challenges.

‖After 7 challenges.

tumors to induce the formation of hemagglutinins (*19*); X-ray treatment was necessary to prevent the absorption of antibodies by the growing tumors.

The results (table 8) show that the variant MCAGA induced the formation of anti-A antibodies in A.CA mice but not anti-A.CA in A. This agrees with our previous observations that variants breeding true in a given parental strain have lost the *H-2* isoantigens specifically derived from the opposite parental strain. So far, the same result has been obtained for all variants tested that were compatible with preimmunized parental mice (*5*, *6*). More variable results were obtained with variants growing in untreated but not in preimmunized mice.

Although both MCAGA lines passed through newborn A.CA mice appeared to be compatible with the A.CA strain, including preimmunized hosts, they maintained their ability to provoke the formation of anti-A antibodies in A.CA mice to an undiminished extent. Thus they have not lost the *H-2* isoantigens derived from the A strain and, serologically, cannot be considered as being truly compatible with the A.CA strain despite their transplantation behavior. The change occurring in these tumors after passage in newborn mice appears to be different from variant formation and is more reminiscent of the behavior of nonspecific tumors growing in all kinds of mice, despite isoantigenic differences and in the presence of homograft reactions. It has been pointed out previously (*19*) that the behavior of such tumors cannot be explained by isoantigenic loss, and the true mechanism of the change remains unknown.

In this experiment, while it was thus impossible to establish variants that had lost all *H-2* isoantigens differentiating the 2 parental strains, it was of particular interest to investigate whether serial passage of a nonspecific, originally A strain variant through A.CA mice can lead to the re-establishment of the missing isoantigenic phenotype, as detected by the formation of anti-A.CA hemagglutinins in A mice. The data in table 8 give a certain indication that this may occur, both in line 43-C, carried in A.CA for 13 passages, and in line 43b-C, established independently and carried in A.CA for 11 transfers. This isoantigenicity, comparatively slight as it was, disappeared completely when line 43-C was carried in A mice, 43-C-A (table 8). From the present data it cannot be decided whether the appearance of A.CA isoantigens in lines 43-C and 43b-C is really due to a re-establishment of the missing A.CA phenotype or to contamination with A.CA stroma cells. More critical experiments are planned to distinguish between these possibilities.

Attempts to Elucidate the Mechanism of Variant Formation—The Possible Role of Immunological Enhancement

Immunological enhancement (*14*), by pretreatment with lyophilized tumor or with isoantiserum, empowers certain tumor homografts to grow in otherwise resistant hosts. Such growth may lead to a "physiological" (*14*) change in the tumor, enabling it to grow for several passages in untreated hosts of the same strain, though as a rule it dies out eventually.

The question arose whether enhancement, known to be mediated by humoral antibodies, is possibly involved in variant formation. In one series of experiments, animals of one parental strain were pretreated immediately before inoculation by 1 intraperitoneal injection of 0.1 to 0.2 ml. isoantiserum directed against the opposite parental strain or with tumor supernatant prepared as follows: Equal volumes of fresh tumor tissue and Ringer's solution were mixed and homogenized in a glass homogenizer. The homogenate was centrifuged at 10,500 × g in a refrigerated Spinco ultracentrifuge. The supernatant was diluted 1:10 with Ringer's solution and stored at −20° C. Three or 4 challenges were given in 0.5 ml. volumes, at intervals of 2 days. Tumor inoculation was performed 2 days after the last challenge.

A total of 20 tumors were inoculated into parental hosts pretreated in this way, but only in 10 cases could we register a clear-cut enhancing effect. The "enhanced" tumors were transplanted further to untreated mice of the same parental strain and to other genotypes. Some of them turned out to be false positives, others were nonspecific, while still others were true variants, specifically compatible with preimmunized mice of the parental strain and capable of breeding true during serial transfer. When considering those neoplasms which could give rise to variants in nonenhanced mice in a certain frequency, it cannot be said that enhancement has facilitated the establishment of true variants to any detectable extent or in any specific way.

More significant, enhancement did not seem to promote the formation of any variant that did not also arise spontaneously in a given F_1 tumor-parental-host system. Neither did it permit the establishment of true variants from homozygous tumors, compatible with a foreign homozygous *H-2* genotype nor of variants from heterozygous tumors, exclusively compatible with a semi-isologous F_1 type derived from an outcross of one parental strain with a third strain.

Another attempt was made to check the possible role of enhancement in the establishment and maintenance of variants, by inoculating them into parental strain mice preimmunized against the opposite parental strain by a single challenge of mixed spleen, liver, and kidney tissue, given intraperitoneally exactly 7 days prior to tumor inoculation. According to Kaliss (*14*), when enhanced tumors are tested in preimmunized mice, they usually fail to grow, at least when the time between the immunizing (first) inoculum and the grafting of the enhanced tumor is short enough (*e.g.*, 7 days). Table 9 shows the results of these tests. It appears that the variants grew equally well in both types of preimmunized mice.

The Possible Role of "Antigen Suppression"

Some experiments were performed to determine whether exposure of the heterozygous F_1 cells to isoantiserums directed against one parental strain might result in the phenotypic suppression of the isoantigens specifically derived from that strain. These experiments were stimulated by the finding of Sonneborn, Schneller, and Craig (*20*), on *Paramecia*, regarding

TABLE 9.—Comparison between the growth of established variants in their parental host

Name of variant	Genotype of origin	Strain of variant compatibility	Growth in preimmunized mice of strain shown in column 3*	
			7-day pre-immuni-zation	Usual pre-immuni-zation
MCADA2	(A × A.CA)F_1	A	3/3	2/3
MCAAA	(A × A.CA)F_1	A	3/3	1/2
MCAGA	(A × A.CA)F_1	A	3/3	3/3
BACAA	(A × A.CA)F_1	A	2/2	3/3
MCABC	(A × A.CA)F_1	A.CA	2/2	3/3
MCAGC	(A × A.CA)F_1	A.CA	3/3	2/2
MCADC	(A × A.CA)F_1	A.CA	3/3	
LNSAA	(A × A.SW)F_1	A	3/3	3/3
MSAIA	(A × A.SW)F_1	A	3/3	5/5
MSWBA	(A × A.SW)F_1	(A × DBA)F_1	3/3	3/4
MSABS	(A × A.SW)F_1	A.SW	6/6	3/3
LNSBS	(A × A.SW)F_1	A.SW	3/3	
MSAGS2	(A × A.SW)F_1	A.SW	6/6	6/6
MSAE	(A × A.SW)F_1	A.SW	2/3	1/3
MSAHS	(A × A.SW)F_1	A.SW	3/3	
MSAIDS	(A × A.SW)F_1	(A.SW × DBA)F_1	3/3	1/1
MSAIS	(A × A.SW)F_1	(A.SW × C3H)F_1	2/3	3/3
MSCGS	(A.SW × A.CA)F_1	A.SW	6/6	5/5
MSCFS	(A.SW × A.CA)F_1	A.SW	3/3	3/3
MSCEC2	(A.SW × A.CA)F_1	A.CA	3/3	2/3

*Preimmunized in the usual way (1 challenge with tumor, containing the isoantigens of the opposite parent, approximately 1 month before testing) or 7 days before inoculation, with normal tissue (spleen, kidney, and liver, given intraperitoneally).

the behavior of certain serotype alleles, which are expressed in heterozygotes (*cf. 21*). Loss of the phenotype corresponding to one allele was repeatedly observed but was not due to the total loss of the corresponding allele, however, since the missing phenotype could be restored by change of temperature. The observations were interpreted as being due to differential expression of alleles in heterozygotes. By analogy, it was speculated that exposure of heterozygous tumor cells to the environment of one parental strain may result in the differential suppression of the isoantigenic products of the *H-2* allele derived from the opposite parental type, *e.g.*, due to the action of antibody. Experiments were therefore performed in which F_1 tumor cells were exposed before each transfer to specific isoantiserums directed against one of the parental strains. The cell suspensions were incubated at 37° C. for 1 hour in the presence of antiserum and complement, followed by subsequent inoculation into both parental strains and into the F_1 hybrid of origin. Untreated control suspensions were tested in the same way. This procedure was carried out serially during 4 and 6 passages, respectively, with the MACC sarcoma and the BNSB carcinoma. Each tumor was carried in 2 treated lines (one serially incubated with P_1-anti-P_2 and the other with P_2-anti-P_1 serum; both lines were treated *in vitro* and carried in the F_1 hybrid of origin *in vivo*). *Both* treated lines gave a larger number of progressively growing tumors in *both* parental strains than untreated inoculums. There was no difference between untreated inoculums derived from the original, untreated line and un-

treated inoculums derived from the antiserum-treated line, which was always carried in the F_1 hybrid of origin, as mentioned previously. Furthermore the takes obtained in the parental strains from the treated inoculums did not breed true in preimmunized mice and could therefore not be classified as true variants. Subsequently it turned out that similar "nonspecific" takes could be obtained in *both* parental strains by treating the cells with complement alone, without antiserum. In 17 of 30 tests, treatment with complement resulted in a higher number of takes in a parental strain than with untreated inoculums. These did not turn out to be true variants on further testing; neither did the complement-treated F_1 line maintain its ability to give rise to an increased incidence of takes in the parental strain on the next passage, if untreated inoculums were used. Further studies are in progress to investigate this effect, but it is clearly different from the formation of specific variants. Also, it was concluded that antiserum treatment *in vitro* has not been successful in inducing the formation of variants.

Treatment With X Rays and Methylcholanthrene

If variant formation would be analogous to ordinary mutations, its frequency could be expected to increase after X-ray treatment. If it were similar to the cellular variation presumably provoked by methylcholanthrene in the course of sarcoma induction, it ought to be influenced by treating the established tumor cells with this compound. Experiments were carried out to investigate these possibilities.

X-ray treatment was previously found (*2*) to increase the takes of the MSWB sarcoma in the parental A.SW strain, from 17 to 49 percent on an average. Since there is a high frequency of spontaneously formed variants with this tumor, other tumors less apt to throw off spontaneous variants were used in the present study. Each tumor was treated *in vivo* with X-ray doses of 100 to 500 r, and then transplanted to the original F_1 type and to both parental strains. Subsequently the tumor received similar treatment before each passage and was thus subjected to serial irradiation for 4 to 15 transfer generations in 3 tumors; a control line from the same original inoculum pool was carried and parallel tests were made with the serially irradiated line. In addition, control data were available from tests on the main line of each tumor. The results are shown in table 10. With the possible exception of small differences, the significance of which is doubtful, X-ray treatment does not seem to have influenced variant formation in these host-tumor systems to any detectable extent.

Intratumoral injection of methylcholanthrene, 0.5 mg. in 0.1 ml. dissolved in trioctanoin, 2 days prior to transplantation was tried with 3 tumor lines. As shown in table 11, this procedure did not promote the formation of variants.

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TABLE 10.—Serial X-ray treatment and variant formation

Tumor	Genotype of origin ($P_1 \times P_2$)	Type	Subline	Number of passages	Treatment	Takes in: P_1	Takes in: P_2
MNSF	(A × A.SW)F_1	MC-sc.	Control	11	—	2/39*	0/45
			Control to X line	4	—	1/28	1/26
			X line	4	300 r 200 r 500 r 200 r	0/35	4/51†
MNSI	(A × A.SW)F_1	MC-sc.	Control	10	—	0/29	1/41‡
			Control to X line	7	—	0/15	14/20
			X line	15	Between 100 and 500 r before each transfer	3/36	20/67
MNSB	(A × A.SW)F_1	MC-sc.	Control	13	—	0/18	1/19
			Control to X line	3	—	0/6	1/20
			X line	8	Between 100 and 500 r before each transfer	0/16	10/47
BYCD	(A.CA × A.BY)F_1	Mammary cancer	Control	9	—	0/30	0/46
			X line	7	150 r before each transfer	0/14	0/15
LNSB	(A × A.SW)F_1	Lymphoma	Control	30	—	2/87	8/98
			X line	15	150 r before each transfer	4/42§	11/39

*One tumor tested further: variant specifically compatible with untreated but not preimmunized A mice.
†One tumor tested further: variant specifically compatible with untreated and preimmunized A.SW mice.
‡Variant specifically compatible with untreated but not preimmunized A.SW mice.
§Two tumors tested further: both were false positives.

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TABLE 11.—Intratumoral methylcholanthrene treatment and variant formation

Tumor	Genotype	Type	Line	Number of passages	Takes in: P_1	Takes in: P_2
					A	A.BY
MAYA	(A × A.BY)F_1	MC-sc.	Control line	7	2/38*	9/31†
			MC-subline	2	0/14	0/10
					A	A.SW
MNSB	(A × A.SW)F_1	MC-sc.	Control line	13	0/18	1/19
			MC-subline	5	1/18‡	1/17§
					A	A.SW
MNSC	(A × A.SW)F_1	MC-sc.	Control line	9	1/23¶	1/33‖
			MC-subline	9	3/31	1/33

*One tumor tested further: false positive.
†One tumor tested further: specific variant, compatible with both untreated and preimmunized A.BY mice.
‡False positive.
§False positive.
‖One tumor tested further: specific variant, compatible with both untreated and preimmunized A.SW mice.
¶One tumor tested further: false positive.

Attempts to Detect Possible Genetic Recombination Between Tumor Cells

To detect the possible occurrence of genetic recombination between different tumor cells, highly specific tumors, *i.e.*, tumors showing little or no tendency to give rise to variants spontaneously, containing different *H-2* markers were mixed in approximately equal amounts. Usually a sarcoma of (A × A.SW)F_1 origin (*H-2^{a}H-2^s*) was mixed with an (A.BY × A.CA)F_1 sarcoma (*H-2^{b}H-2^f*). Altogether 24 such experiments were performed. The mixture was inoculated subcutaneously into newborn DBA/2 (*H-2^{d}H-2^d*) or C3H (*H-2^{k}H-2^k*) mice. These hosts were chosen with the intention of using immature animals as inactive as possible in their homograft reaction. Since their *H-2* constitution was different from both hybrid tumors and their possible recombinants, it was hoped that negative selective pressures against the latter might be avoided or minimized in this way. The mixed inoculums developed into tumors in nearly 100 percent of the newborn mice, after approximately 2 weeks. The tumors were tested in the original (A × A.SW) and (A.BY × A.CA)F_1 hybrid types. Both components of the original mixture could be recovered and subsequent tests showed that one passage through the genotype of origin was usually sufficient to separate the original tumors in pure form. There was no evidence for any change in specificity due to this procedure, and the component tumors did not seem to develop an increased ability to give rise to variants in the parental strains.

The mixed tumors removed from the infantile DBA or C3H mice were also tested for possible specific recombinants, selectively compatible with F_1 hybrids of the types A × A.BY (*H-2^{a}H-2^b*), A × A.CA (*H-2^{a}H-2^f*), A.SW × A.BY (*H-2^{s}H-2^b*), and A.SW × A.CA (*H-2^{s}H-2^f*). A few progressively growing tumors were obtained in some, but these turned out to be "false positives," incapable of continued growth in the same geno-

type on serial transfer, or variants of the conventional type, compatible not only with a new F_1 hybrid type but also with one of the parental strains of the F_1 and all other F_1 combinations in which the same parent has entered the cross. No clear-cut evidence could be obtained to show the occurrence of genetic recombination, since no new types were obtained that would have been specifically and exclusively compatible with a new F_1 hybrid combination. However, negative experiments do not exclude the possibility of recombination and this work is therefore being continued under varied experimental conditions and with different tumors.

DISCUSSION

The possible mechanism of variant formation has been discussed in detail in previous publications (*5*, *6*) and in a paper by Hellström (*8*). This discussion may be restricted to a consideration of some aspects emphasized by the present paper.

Studies on a large number of F_1 tumors have confirmed and emphasized the previous conclusion that variant formation follows an individually different pattern in different tumors, even if they have been induced by the same agent in the same tissue of the same genotype and have a similar morphology. No over-all differences could be discerned between the 3 categories of neoplasms studied in detail so far: methylcholanthrene-induced sarcomas, mammary carcinomas, and lymphomas. A fourth category, estrogen-induced and dependent testicular tumors, has not given any variants so far, but the number is too small to decide whether this is due to chance or a regular characteristic of this tumor type in particular or hormone-induced tumors in general.

If it is accepted that at least some cases of tumor progression, *i.e.*, the stepwise development of certain tumors toward increased malignancy (*22*), are due to variation and selection (*cf. 23*), variant formation can be considered as a model of progression (*6*). Variant formation can be viewed as the loss of isoantigens preventing growth in a foreign host genotype, while progression is essentially a gradual loss of responsiveness to various growth-controlling homeostatic forces in the autochthonous host. In a sense, variant formation can be regarded as an experimental model, even though an unnatural, synthetic one, of the deletion theory of carcinogenesis. Bearing the superficial nature of this parallel in mind, it is nevertheless interesting that there is a certain analogy between the random and unpredictable nature of a certain progressional step in different tumors of the same type (*22*, *24*), and the varying probability of the formation of a given variant in different tumors of the same type. It is also of interest that all variants so far isolated are of the *loss* type, and no new types involving changes to an alternative state could be isolated in semi-isologous F_1 hosts. This seems to exclude point mutation and transduction as possible mechanisms and focuses attention on somatic crossing over or gross chromosomal changes, if the mechanism of variation is to be sought

at the genetic level. The genetic assumption would be well in accordance with the increased specificity of the variants, pointing toward a homozygous rather than a hemizygous state of the variants, *i.e.*, somatic crossing over rather than chromosomal losses, and their irreversibility during prolonged passage in the genotype of origin. It is weakened by the negative results of the experiments where neither X rays nor methylcholanthrene could promote variant formation. X rays were found to increase variant formation in the MSWB tumor used in a previous study (*2*) and similar results were obtained by Dhaliwal (*25*) who treated a methylcholanthrene-induced sarcoma arising in an (A × A.SW)F_1 mouse by X rays prior to inoculation. He has also seen an increased variant formation after treatment of the tumor-cell suspensions with triethylene-melamine (TEM).

A series of attempts to "switch" established variants to the opposite type have failed. On the genetic hypothesis, this would be in agreement with a homozygous variant cell derived from somatic crossing over. It cannot be excluded, however, that a cell losing both of its *H–2* factors may become inviable and thus escape detection. An apparent exception to this rule, in which transplantation results indicated that a loss of both *H–2* alleles may have occurred, turned out on closer analysis to be due to the development of a "nonspecific" subline, not characterized by a second isoantigenic loss. The growth of "nonspecific" tumors in foreign genotypes has not been satisfactorily explained and it does not have to be accompanied by isoantigenic loss (*19*). It seems to be a phenomenon different from the formation of specific variants (*5*, *6*).

Variants could only be obtained from heterozygous but not from homozygous tumors and their selective compatibility corresponded to one of the parental strains. No variant could be isolated that would have been exclusively compatible with another, semi-isologous F_1 hybrid type. The variants were stable and the missing isoantigenic phenotype could not be re-established upon prolonged passage in the F_1 hybrid genotype of origin. They were often more specific than the original tumor from which they had been derived and they could never be "switched over" to the opposite parental type by passage in newborn mice, or made to lose their residual *H-2* isoantigens, corresponding to their compatible parental type. If it is assumed that variant formation is due to a change at the genetic level—and this cannot be decided conclusively at present—these results are best interpreted as somatic crossing over, leading to the establishment of homozygous variants corresponding to the 2 parental types.

Immunological enhancement did not promote the formation of variants in any significant way. It was not possible to "suppress" *H-2* isoantigens by antiserum treatment in the presence of complement; a certain increase of "nonspecific" takes was obtained after this treatment, not accompanied by antigenic loss and not perpetuated by serial transfer. The same result could be obtained by treating the cells with complement alone.

In the present experiments X-ray or methylcholanthrene treatment of established tumors did not influence variant formation. No evidence of

genetic recombination was obtained in experiments when 2 F_1 tumors of complementary types were grown together in mixture in "neutral" newborn hosts.

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Chromosomes in Primary and Transplanted Leukemias of AKR Mice[1]

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SUMMARY

Chromosome analyses were performed on leukemias of strain AKR mice, mostly on spleen but also on thymus, lymph nodes, and lymphosarcomas. Spontaneous primary leukemias in a comparable stage of development showed a varying proportion of cells with aneuploid chromosome numbers, with a usual range from 39 to 42, but also there were cells with 43 and 44 chromosomes in 4 animals. Most of the 17 mice with primary leukemias, and 10 with leukemias and tumors resulting from transplantations, had a modal chromosome number of 40 (diploid). In 5 animals with primary leukemias the mode was 41, and 1 had 2 modes, 42 and 43. More than 80 percent diploid cells were found in 7 mice with primary leukemias and in 6 with leukemias or tumors resulting from transplantations. The chromosome morphology of the diploid cells was generally normal, though in 2 animals with predominantly diploid leukemias, several cells (8 and 18%) had a small marker chromosome. The predominance of diploidy was maintained during continued transplantation. This demonstrates that neoplastic cells can have a diploid chromosome complement without detectable abnormalities.—J. Nat. Cancer Inst. 25: 295–305, 1960.

MANY MAMMALIAN tumors consist of cells having an altered genetic composition, which can be transmitted over a series of cell generations. Changes in chromosome structure, in chromosome number, and in amount of deoxyribonucleic acid (DNA) have been reported in transplanted tumors (*1–13*) and in neoplasms induced by chemicals (*11*, *12*) and radiation (*13*). Similar cytological changes were usually noted in spontaneous carcinomas (*14–23*). In this connection it appeared of interest to study the chromosome complement of neoplastic cells of spontaneous leukemias. The mammary carcinomas of C3H and DBA mice (*24*) and papillomas and carcinomas of rabbits (*25*) are the only tumors caused by a virus or virus-like agent that have been examined cytologically so far. Neoplastic cells of 16 out of 19 mammary carcinomas studied had a predominantly diploid chromosome number (*24*). This result raises the question whether chromosomal alterations are absent or rare in virus-induced tumors. The present study of spontaneous leukemias of AKR

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mice was undertaken to gain information concerning this problem. The leukemias of AKR mice seemed suitable because the histopathology is well known (*26–30*).

MATERIALS AND METHODS

The strain AKR mice used throughout the experiment were obtained from Millerton Research Farm, from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and from our laboratory.

Transplantation was performed by implanting small pieces, not exceeding 2 mm., of spleen, lymph nodes, thymus, or thymic lymphosarcoma subcutaneously in both axillary regions of young AKR mice, usually 4 weeks old. If a chromosome count of the transplanted tissue was desired, a part of the tissue was extirpated before the application of colchicine. Cervical lymph nodes were generally used for such purposes since they were easily obtained without a major operation. Spleen tissue was obtained by applying a ligature and removing one quarter to one third of the organ.

Material for cytological studies was prepared according to the method of Ford and Hamerton (*31*). Colchicine was injected intraperitoneally 2 hours before the animals were killed. Small pieces of tissue were chopped and pretreated in hypotonic sodium citrate solution for 20 to 30 minutes at 37° C. One part of the pretreated tissue was fixed in acetic alcohol (1:3) and stained with the Feulgen reaction for permanent squash preparations, and another part was placed in acetic orcein for squash preparations.

RESULTS

Normal Tissue

The chromosome number in spleen and thymus of 4-day-old AKR mice was determined to establish variation in normal tissues. This was necessary to assess the significance of chromosomal alterations in leukemic tissues. The results are summarized in table 1. The chromosome numbers of hepatic cells of a regenerating liver after a partial hepatectomy were added to show the variation in a tissue in which a high mitotic rate was induced experimentally. The chromosome counts on regenerating liver were performed 2 days after partial hepatectomy of 4-month-old mice. Cells with a smaller chromosome number than the diploid one were present in a relatively small percentage of all tissues examined. This one-sided distribution of the counts agrees with that obtained on normal reticular tissue of mice (*13*, *32*). The simplest interpretation is to assume that a loss of chromosomes during preparation, and an overlapping of chromosomes in insufficiently spread metaphase plates, causes underestimation of the true diploid number. Several other possibilities such as elimination of chromosomes and nondisjunction must be taken

into consideration, though they appear unlikely (*13*). Cells having 41 or more chromosomes were not observed in normal tissue of young AKR mice, which again agreed with data reported for other strains.

TABLE 1.—Chromosome numbers of normal tissues

Organ	Age	Number of cells analyzed	Percentage of cells with indicated chromosome number						
			36	37	38	39	40	41	42
Spleen	4 Days	50				6	94		
Spleen	6 Weeks	60				3	97		
Thymus	4 Days	55				2	98		
Regenerating liver	4 Months	46	2	4	4	7	81		2

Spontaneous Primary Leukemia

Chromosome number and structure were examined in spleen cells of 16 leukemic AKR mice with greatly enlarged spleen, cervical, and mesenteric lymph nodes. Histologic examination showed the liver to be infiltrated in the mice studied. The thymus was usually greatly enlarged, sometimes filling a large part of the chest cavity. The presence of leukemia in the tissue examined was checked by transplanting small pieces of spleen, lymph nodes, or thymus subcutaneously into young nonleukemic AKR mice. In all cases the grafted tissue caused a lymphosarcoma at the site of transplantation, a general leukemia, or both, within 10 to 15 days, a period when spontaneous leukemias are rare. Tissue of 6-week-old nonleukemic mice was grafted as a control. The results are presented in table 2.

TABLE 2.—Graftings of leukemic and nonleukemic AKR tissue

Mice	Organ grafted	Number of animals	Grafts*		
			Lymphosarcomas	Leukemia	Lymphosarcoma and leukemia
Nonleukemic	Thymus	12	0 (180)	0 (180)	0 (180)
	Spleen	12	0 (180)	0 (180)	0 (180)
Leukemic	Thymus	5	4 (28–75)	1 (10–40)	0
	Spleen	9	0	9 (18–23)	0
	Lymph nodes	44	16 (30–63)	20 (14–30)	8 (14–24)

*The figures in parentheses indicate the time interval, in days, between inoculation and death of the mice.

The chromosome counts obtained on spleen cells of leukemic mice, summarized in table 3, show a marked numerical variability ranging from a mixed aneuploid cell population to a population consisting only of diploid and a small number of subdiploid cells. The chromosome counts obtained in different organs of 2 leukemic mice are given in table 4.

TABLE 3.—Chromosome numbers of spontaneous leukemias in spleen of AKR mice

Mouse number	Number of cells analyzed	Percentage of cells with indicated chromosome number						Marker chromosomes
		39	40	41	42	43	44	
22	32	6	94					None
21	28	10	90					None
19	21	5	95					None
3	50	—	96	4				None
20	70	7	90	3				Common
18	33	3	91	6				Common
14	71	5	72	21		2		None
16	49	10	63	23	4			Rare
2	59	3	68	27	2			Rare
23	30	3	67	23	7			None
9	30	—	17	70	13			None
10	58	—	14	83	3			None
12	50	2	12	80	6			Rare
5	53	—	6	38	34	22		Common
7	45	2	22	65	2	7	2	Common
1	68	—	22	4	30	34	10	Common

TABLE 4.—Chromosome numbers of different organs of spontaneous leukemias in AKR 19 and AKR 14

Mouse number	Organs	Number of cells analyzed	Percentage of cells with indicated chromosome number				
			39	40	41	42	43
AKR 19	Spleen	21	5	95			
	Thymus	32	6	81	13		
	Cervical nodes	41	9	88	3		
AKR 14	Spleen	71	5	72	21		2
	Thymus	55	9	73	15	3	
	Cervical nodes	20		80	20		

Chromosomes of distinct morphology were present in several of the aneuploid and diploid cells of leukemic spleen and thymus. Two examples of extremely small chromosomes in diploid cells are shown in figures 1 and 4. One of the small "marker" chromosomes occurred in 6 percent of the diploid spleen cells of AKR 18 and the second one was found in 18 percent of the cells examined of AKR 20. Distinct marker chromosomes were observed in the aneuploid cells of AKR 16, 2, and 12. An example is shown in figure 2. This particular chromosome was generally more intensely stained, a property easily observable at the prometaphase stage or in metaphase plates with less contracted chromosomes. The additional chromosome in cells containing 41 chromosomes could not be distinguished in AKR 23, 14, 9, and 10 (fig. 3). It can be assumed that the real number of cells containing small "marker" chromosomes is higher than that observed since they may escape detection in many of the metaphase plates with highly contracted chromosomes. No aberrations in chromosome morphology were observed in leukemic spleen of AKR 22, 21, 19, and 3. Greater morphologic differences were observed in the spleens of AKR 5, 7, and 1, which consist of a mixed aneuploid cell population. An evalu-

ation of these alterations was tried by measuring the length of the chromosomes and arranging them in an idiogram. Two samples have been reported (*33*).

Transplanted Leukemias

The behavior of leukemic cells with diploid chromosome numbers in subsequent transplant generations appeared of particular interest. The question was raised whether a small number of aneuploid cells would overgrow the diploid ones in the grafted lymphosarcomas. Emphasis was therefore placed on an accurate examination of transplanted leukemias or lymphosarcomas derived from leukemic tissue with predominantly diploid cells. Two examples are given of chromosome counts obtained from subcutaneous lymphosarcomas and of leukemic spleens of the first transplant generation (table 5), and from lymphosarcomas of the third, fifth, and seventh transplant generation (table 6) where small pieces of lymphosarcomas from mouse #1 of each transplant generation were used for further transplantation. The chromosome numbers of the leukemic

TABLE 5.—Chromosome numbers of spontaneous leukemia and the first transplant generation of AKR 18 and AKR 14

Tissue grafted	Number of cells analyzed	Percentage of cells with indicated chromosome number			
		39	40	41	42
AKR 18					
Primary					
Cervical nodes	52	9	85	6	
First generation					
Tumor (mouse #1)	45	11	89		
Spleen (mouse #2)	31	3	97		
AKR 14					
Primary					
Cervical nodes	21		81	19	
First generation					
Spleen (mouse #1)	40	7	75	15	3
Tumor (mouse #2)	38		69	26	5
Spleen (mouse #2)	20	10	75	15	
Tumor (mouse #4)	53	3	57	27	13
Spleen (mouse #4)	21	9	77	14	

TABLE 6.—Chromosome numbers of spontaneous leukemia (AKR 15) and transplanted subcutaneous lymphosarcomas

	Number of cells analyzed	Percentage of cells with indicated chromosome number		
		39	40	41
Primary				
Cervical nodes	34	5	89	6
3 Generations				
Mouse #2	28		93	7
Mouse #3	28	4	75	21
5 Generations				
Mouse #1	28	3	93	4
Mouse #2	24	4	83	13
7 Generations				
Mouse #3	19	10	85	5

tissue used for the first graft are also given in tables 5 and 6. The diploid cell population was not overgrown by aneuploid cells in any of the specimens examined, though the proportion between the cell lines appeared to be altered in several instances. The number of aneuploids increased from around 6 to 21 percent in the lymphosarcoma of mouse #3 (table 6), whereas they apparently decreased in the tumor of mouse #1 and the spleen of mouse #2 (table 5). A minute chromosome appeared in only one case, in the cells with 41 chromosomes of mouse #3 of the third transplant generation.

DISCUSSION

The 27 mice studied with leukemias or lymphosarcomas showed a considerable variation in number of aneuploid cells present, in spite of the similarity of the leukemic stage, as judged by the enlarged thymus, spleen, and lymph nodes and the histologic appearance of these organs. A mixed population containing cells with diploid and aneuploid (41, 42, and 43) chromosome numbers was present in the spleen, lymph nodes, and thymus of several mice (tables 3 to 5). In several animals all or nearly all cells were observed to have an apparently normal chromosome complement.

The inconsistency of these chromosome patterns makes interpretation extremely difficult. Arguments can be put forward that cytologically undetectable changes of chromosome structure were present in all leukemic cells with an apparently normal diploid chromosome complement. Translocations not including centromeres and not altering the chromosome length beyond the largest and the smallest chromosome could easily escape detection. Doubt could also be expressed concerning the malignant nature of the diploid cells. However, a transplantation of these cytologically normal cells resulted, in all cases examined, in a rapid formation of subcutaneous solid tumors, a general leukemia, or both, without any visible alterations of the apparently normal chromosome complement. The chromosome number remained predominantly diploid over 7 further transplant generations studied. It must therefore be concluded that leukemic cells can have a diploid chromosome number with no detectable chromosome abnormalities.

On the other hand it is difficult to ignore the aneuploid cells of several leukemic mice. Their presence in spleen, lymph nodes, and thymus must be related to the leukemic stage since they were absent in normal animals of the same strain. The possibility that these aneuploid cells have been formed during metastases and do not represent the original neoplastic cells should be considered. An interpretation is made more difficult by the lack of information concerning the origin of leukemic cells. Although a number of experiments point to a predominant role of the thymus, exact proof of the thymic origin of leukemic cells is lacking (*34-37*).

The presence of leukemic mice with diploid and with aneuploid chromosome complements raises the question whether differences in the etiology

of leukemia might exist. Significant differences seem to exist between cells in acute and chronic leukemias of man, the former being aneuploid or diploid with chromosomal alterations, and the latter having a normal diploid chromosome complement (*38*).

Neoplastic cells with a normal chromosome set occur in mammary carcinomas of C3H and DBA mice (*24*) and in papillomas of rabbits (*25*). The spontaneous leukemias and the transplanted lymphosarcomas described can be added to the tumors consisting predominantly of a diploid cell population. Common to most of these tumors is their probable induction by viruses or virus-like agents. In contrast, neoplasms induced by chemical carcinogens or spontaneous tumors of apparently nonviral origin are characterized by aneuploid chromosome numbers (*10*), aneuploid DNA values (*22, 23*), and chromosomal abnormalities (*16, 19*). It appears likely, though unproved, that this difference is due to the different method of tumor induction. The genetic change of neoplastic cells might be caused by introducing a new genetically active agent such as a virus, or by altering the genome of the cell by producing aneuploid chromosome complements or chromosomal rearrangements.

A similarity between spontaneous and radiation-induced leukemia is evident if the results described are compared with those obtained by Ford *et al.* (*13*). Some of the reticular neoplasms consist of predominantly aneuploid cells and some contain a chromosome complement undistinguishable from a normal diploid cell in both instances. This similarity appears of great interest considering the different types of induction. The production of chromosomal and mitotic changes by X rays is a well-known phenomenon which can result in the abnormal aneuploid chromosome complements of leukemic cells. It is not known at present whether carcinogenic viruses cause chromosomal aberrations or if they affect chromosomes at all. Possibly the first neoplastic cells caused by viruses are diploid and only during the following rapid cell divisions do aneuploid cells occur at random and overgrow the cell population.

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PLATE

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PLATE 26

FIGURE 1.—Metaphase with 40 chromosomes from leukemic spleen AKR 18. One marker chromosome. × 4,400

FIGURE 2. Metaphase with 41 chromosomes from leukemic spleen AKR 12. One marker chromosome. × 4,400

FIGURE 3.—Metaphase with 41 chromosomes from leukemic spleen AKR 14. × 4,400

FIGURE 4.—Metaphase with 40 chromosomes from leukemic spleen AKR 20. One marker chromosome. × 4,400

FIGURE 5.—Representative area of leukemic thymus AKR 14. Hematoxylin and eosin.

1

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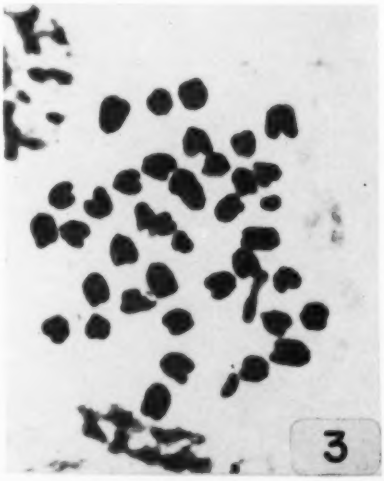
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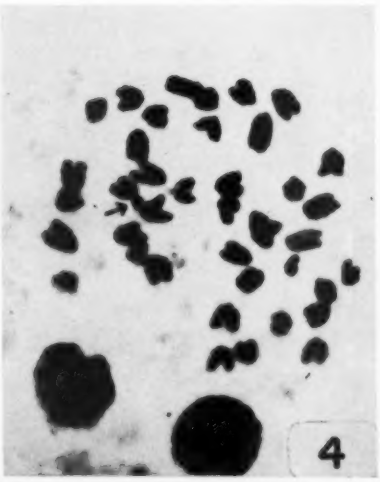
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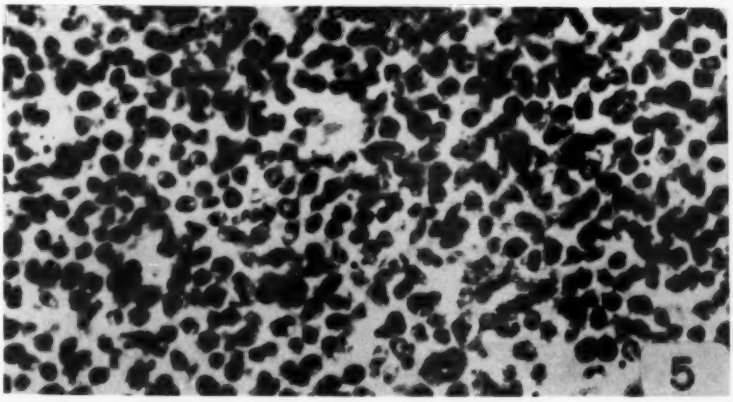
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Contact Transmission of Rous Sarcoma [1]

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SUMMARY

In 2 experiments involving a total of 820 inbred White Leghorns, the transmission of Rous sarcoma virus under conditions of direct bird-to-bird contact, which resulted in Rous sarcomas of the subcutis, the skeletal muscle, and the viscera, has been observed for the first time. Diluted virus applied to open feather follicles caused tumors, suggesting a mode of transmission; however, this may not be the only one, since almost half of the contact-positive chickens had tumors in the viscera only.—J. Nat. Cancer Inst. 25: 307–313, 1960.

THE ROUS sarcoma virus (RSV) (*1*) has long been considered noncontagious. In 1912 it was reported (*2*) that susceptible chickens did not develop tumors when they were fed tumor tissue or were allowed to intermingle for long periods with chickens bearing Rous sarcomas. There have been no reports since which differ from these observations (*3–7*).

In recent studies with RSV involving a long experimental period, a high proportion of uninoculated inbred White Leghorns developed myxofibrosarcomas as a result of being maintained in contact with inoculated chickens of the same hatch. Tumors in contact birds were grossly and microscopically indistinguishable from the Rous tumor.

MATERIALS AND METHODS

The RSV obtained from Dr. W. Ray Bryan, of the National Cancer Institute, was part of a standard virus preparation identified as CT750. It was a microsome fraction derived from chicken-tumor tissue (*8*). The virus was stored in a dry-ice chest at about −80° C. until just before inoculation. All dilutions were made with physiological saline containing 2 percent normal horse serum, except where otherwise noted.

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Single-comb White Leghorns of inbred line 15I, selected for susceptibility to lymphomatosis and maintained in isolation for the preceding 18 years, were used. This strain was also quite susceptible to RSV (*9*). Inoculations were made at 9 days of age by the intravenous (cubital vein) or the subcutaneous (left wing web) route, with 0.2 or 0.1 ml. of inoculum, respectively. All chickens were individually identified by aluminum bands, which were sealed into the web of the right wing when they were inoculated and/or assigned to the various lots.

All chickens were raised in special 3 × 6 foot cubicles with solid walls 4 feet high and ¾-inch mesh wire floor located 1 foot above the concrete floor of the pen. At about 11 weeks of age the chickens were transferred to large pens. Procedures of care and maintenance were similar to those described previously (*10*). Uninoculated chickens, "contacts," were raised in the same cubicle and allowed to intermingle with inoculated chickens of the same hatch.

For one experiment a cubicle was divided in half through its long axis by a double fence made up of 2 lengths of ½-inch mesh wire screen placed ¾ of an inch apart. Inoculated chickens were placed on one side and uninoculated chickens, "indirect contacts," on the opposite side of the fence. This arrangement allowed free interchange of air and suspended particles between the 2 sides but prevented all direct chicken-to-chicken contact. Uninoculated chickens raised in cubicles by themselves were identified as controls. These cubicles were adjacent to and in the same pen with others that contained inoculated chickens. Thus the physical conditions allowed an interchange of air between cubicles with controls and those with inoculated chickens. Furthermore, when chickens were moved to pens, those from different cubicles were allowed to mix.

The birds were examined at periodic intervals for evidence of tumor growths and postmortem examinations were made on all chickens. In most instances the diagnoses were based on gross findings. Microscopic studies of tumor and other tissues were made on a limited sample of positive birds of the contact lots and of a few of the inoculated lots to provide an adequate basis for the diagnostic criteria and an appraisal of the tumors obtained.

RESULTS

The essential data relative to the mortality resulting from Rous sarcomas in inoculated, contact, and control chickens of 2 experiments are presented in table 1.

In the first experiment, 35 of 45 uninoculated chickens raised in direct contact with others inoculated subcutaneously died with Rous sarcomas. In addition, of 59 in contact with 118 chickens inoculated intravenously with a low dose of RSV, 6 developed tumors. There was no overlap in time-to-death between the inoculated and uninoculated chickens. Thus, those inoculated with a 10^{-4} dilution died in 14 to 19 days; those in contact

TABLE 1.—Mortality from Rous sarcomas in chickens inoculated and in those in direct contact

Treatment	Number of chickens	Mortality with tumors		Postinoculation (*days to death*)	
		Number	Percent	Range	Median
Experiment 1					
Cubicle 1*					
Inoculated subcutaneously —4.0 logs dilution	49	46	94	14–19	17
Contact, with subcutaneous inoculation	45	35†	78	37–128	49
Cubicle 2*					
Inoculated intravenously —5.3 logs dilution	118	108	92	15–38	23
Contact, with intravenous inoculation	59	6†	10	50–165	62
Cubicle 3					
Controls	49	0	0	—	—
Experiment 2					
Cubicle 1*					
Inoculated subcutaneously —4.0 logs dilution	59	57	97	16–25	19
Contact, with subcutaneous inoculation	58	23	40	42–104	60
Cubicle 2*					
Inoculated intravenously —6.3 logs dilution	178	99	56	19–100	35
Contact, with intravenous inoculation	57	3	5	62–134	84
Cubicle 3‡					
Inoculated subcutaneously —4.0 logs dilution	50	47	94	17–25	20
Indirect contact, with subcutaneous inoculation	50	0	0	—	—
Cubicle 4					
Controls	50	0	0	—	—

*The 2 lots were maintained in the same cubicle and allowed to intermingle.
†One bird developed a dermal tumor which regressed.
‡The 2 lots were maintained in the same cubicle but mixing prevented by a double-wire fence.

died in 37 to 128 days, and chickens inoculated with a $10^{-5.3}$ dilution died in 15 to 38 days—and their contacts died in 50 to 138 days. None of 49 control chickens developed tumors.

In the second experiment, 23 chickens in contact with 58 inoculated with 10^{-4} dilution died of tumors, and 3 of 57 in contact with those inoculated intravenously with $10^{-6.3}$ dilution died with tumors. None of 50 "indirect contact" chickens showed any evidence of tumorous growths. Controls raised in a separate cubicle also remained free of tumors.

The transmissibility of 2 tumors obtained from "contact" chickens was tested. The first tumor was large, soft, and hemorrhagic, while the second was small, firm, and solid. Five percent saline extracts were processed in a Virtis homogenizer and then centrifuged for 20 minutes at 3000 rpm. Inoculations were made subcutaneously into 2-week-old chickens. Of 20 chickens inoculated with extract of the first tumor, 18 developed tumors and 14 died within 8 weeks. Extract of the second tumor induced neo-

plasms in only 8 of 20 inoculated chickens. Such variation in apparent virus content and its relation to the character of the tumor has been previously reported (*11*).

Tumors grossly visible in the contact birds were located in almost all parts of the body. About one third had only one tumor involving the skin and/or subcutis and the underlying muscle. They were found on various parts of the head, neck, wing, breast, back, and leg. One fifth of the affected birds had external tumors and tumors in the viscera. It was exceptionally interesting that almost one half of all contact-affected birds had grossly visible tumors located only in the viscera. Organs most often affected were the lung, mesentery, spleen, liver, and kidney.

Examinations of sections of tumors obtained from contact birds and from chickens inoculated with extract from contact tumors showed that the variation in morphology was similar to that described for the Rous sarcomas (*12*). There was considerable variation in the relative proportion of the primary constituent of the tumor. Small, firm tumors were largely made up of spindle-shaped fibroblasts with scattered small and large, round cells. The soft, rapidly growing tumors were often hemorrhagic and ulcerated. These contained, in addition to the typical fibroblastic deposits, areas of large, round cells, necrosis, and myxomatous material.

DISCUSSION

The data of the 2 experiments gave overwhelming evidence that the Rous sarcoma was transmitted to uninoculated chickens when they were raised in direct contact with others of the same hatch inoculated by the subcutaneous or intravenous routes. The amount of contact transmission was much greater in cubicles containing chickens inoculated subcutaneously with 10^{-4} dilution of virus, than in those inoculated intravenously with $10^{-5.3}$ and $10^{-6.3}$ dilution. Whether the difference in contact transmission was related to the route or the dosage difference cannot be ascertained from these experiments; quite likely it is related to both factors. Since contact transmission was an unexpected outcome, the experiments were not designed to identify factors influencing the extent or quantity of transmission.

It is quite apparent that the experimental period required for the development of tumors and resultant death is much longer in the contact chickens than in those inoculated. The first tumor death in the contact chickens occurred 37 days after penmates were inoculated, in comparison with 14 days in the inoculated chicks. The median time to death of the former lot was 49 days and the latter 17 days; other inoculated and contact groups showed similar divergent death times.

A part of this difference can be accounted for by the probability that the time of infection of the contact groups was at some period after the time of inoculation. It would appear that the virus must propagate in the inoculated chicken and attain an as yet undetermined level before contact transmission occurs. That the level of virus attained may be

related to the dose is suggested by the data of others (*11*, *13*), who found that the concentration of virus in a tumor was directly proportional to the inoculating dose. Thus the exposure dose of chickens in contact with chickens inoculated with a low dose would be much less than those in contact with chickens inoculated with a high dose because not only did the tumors develop at a slower rate and in fewer chickens but the tumors that did develop no doubt had a lower concentration of virus. This difference in the contact exposure dose is, in fact, suggested by the postinoculation time to death when the known relationship between dose and the median latent period is considered (*14*, *15*).

Research on the possible natural modes of transmission of the Rous or Rous-like viruses has been limited. Intranasal instillation of Rous virus has caused tumors in more than half of the exposed chickens; however, no tumors resulted when chickens were confined to a small enclosure and made to breathe a high-dose aerosol of the same virus for 5 minutes every other day for 5 days (*16*). In experiments at this Laboratory, no tumors resulted from placing 0.5 ml. of a 1:5 dilution of RSV under the tongue of susceptible chickens or when it was applied with a camel's-hair brush to a nonfeathered area of intact skin. However, when it was similarly applied to skin that had been traumatized, either by scratching with the point of a hypodermic needle or by the extraction of immature feathers, tumors occurred in 80 percent of the chickens. In a subsequent experiment, the CT750 virus was diluted to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} with 0.05 M citrate buffer containing ½ percent hyaluronidase, and groups of 20 chickens were exposed by applying with a brush a dilution of virus to a small area of plucked skin.

The first 3 virus dilutions caused tumors at the point of application in over 90 percent of the chickens and the 10^{-4} dilution caused tumors in 70 percent in 18 days. These results indicate that the RSV can easily gain entrance through a feather follicle and cause local as well as visceral tumors. This method of infection may very well be the result of the fortuitous contact of an open feather follicle with an ulcerated tumor or other contaminated surfaces. However, this may not be the only method involved, since almost half of all contact chickens that died with Rous sarcomas showed no evidence of external tumors. Inasmuch as the subcutaneous tissue is very sensitive to the virus, one would not expect the virus to enter the chicken through the skin without causing tumors in the subcutis.

Although the oral (or gastrointestinal) and respiratory routes are obvious possible modes of entry, experimental data available indicate that these routes were probably not involved in transmission resulting in tumors under condition of the contact experiments reported herein. It should be emphasized that no attempt was made to detect the occurrence of inapparent infection which may well have been present in some of the direct and indirect contact chickens.

Why this was the first observation of contact transmission of RSV that resulted in tumors is not understood. There is no question that the biologi-

cal characteristics of the virus can and do change (*17*, *18*). The apparent acquisition of contagiousness may be due simply to an increase in the concentration of the virus; however, variations in the contagious quality of many viruses have been observed. Apparent variations of this type have been observed with a related virus which causes visceral lymphomatosis (*19*). Of equal importance in this system is the susceptibility or sensitivity of the host to respond to the virus. Chickens of the inbred line 15I, which was the source of birds used in these experiments, have been shown to be uniformly suceptible to RSV when inoculations are made by the subcutaneous, intracerebral, and intravenous routes (*9*, *20*). They were also found susceptible when exposed by inoculation or by bird-to-bird contact to viruses causing several forms of leukosis and related tumors (*9*, *18*, *21*, *22*), and finally, tests conducted by several investigators (*20*, *23*) indicate that this stock has been maintained for several years without RSV neutralizing antibodies.

ADDENDUM

After this manuscript was submitted, additional data were obtained which indicate that the virus recovered from the contact-induced sarcomas was immunologically similar to that in the standard Rous sarcoma virus preparation, CT750, used for inoculation. Of 51 adult chickens inoculated subcutaneously with undiluted extract of a tumor resulting from contact with inoculated chickens, 26 developed tumors and 11 of these died. Four weeks after the first inoculation all survivors were again inoculated and only 3 developed tumors. Five weeks after the second inoculation all survivors were challenged with the standard CT750 virus preparation and only 1 developed a tumor, whereas tumors appeared in 23 of 24 controls from the same source as the 51 immunized chickens, but the controls had received no previous inoculations.

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Electrophoretic and Morphologic Studies of a Transplantable Reticulum-Cell Neoplasm in Mice Inducing Amyloidosis [1, 2]

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SUMMARY

The first 10 transfer generations of a reticulum-cell neoplasm of the mouse constantly associated with the development of amyloidosis are described. Microscopic examination revealed that the grafts were pyroninophilic, plasma-cell differentiated reticulum-cell sarcomas, spreading diffusely, especially to the lymph nodes, liver, and spleen. Serum-protein changes observed in paper electrophoresis and immunoelectrophoresis were a hypergammaglobulinemia changing to an increase in the β- (β_2-III) and α_2-fractions ($\alpha_{2\text{-I}}$ and $_{2\text{-II}}$) and eventually to a reduction in albumin and β-globulin and a further increase in the α_2-fraction ($\alpha_{2\text{-III}}$ and $_{2\text{-V}}$); the latter changes were accompanied by urinary excretion, particularly of albumin and β-globulin ($\beta_{2\text{-I}}$). During tumor growth a relative and often an absolute granulocytosis and monocytosis were seen. Amyloid development first was found, most frequently and markedly, in the spleen, then in the liver, in the kidneys, and, occasionally, in many other organs. Mice with the longest survival time showed the heaviest degree of amyloidosis. Our findings indicated that amyloidosis was due to a persistent hyperimmunization, in accordance with the theory of the pathogenesis of amyloidosis advanced by Teilum. The last stage of the serum-protein changes and the urinary elimination of protein were explained as caused by the development of a nephrotic syndrome caused by renal amyloidosis. In mice with a short survival time and extensive tumor growth without amyloidosis, a paraprotein-like γ-protein was found in serum and the tumors showed a more plasma-cell differentiated picture.—J. Nat. Cancer Inst. 25: 315–349, 1960.

SPONTANEOUS DEVELOPMENT of amyloidosis occurs sporadically in old mice (*1*). Experimentally amyloidosis has been induced by various means, such as repeated injections of bacteria (*2*), antigens (*3*), sodium ribonucleate (*4*), pentose nucleotides (*5*), and particularly sodium caseinate (*6–11*). Painting with mineral oils that are more or less carcinogenic has also been shown to induce amyloidosis, whether the paintings result in tumor development or not (*12*). Amyloidosis in mice carrying spontaneous or transplanted tumors has been reported by several authors. Parsons (*5*) found amyloid development in the liver and spleen of mice

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bearing sarcomas serially transplanted for generations; and Dunn (personal communication) observed that growth of a certain transplantation line of fibrosarcoma was associated with amyloid development.

We have studied a transplantation line originating in a reticulum-cell neoplasm which proved to be constantly associated with the development of amyloidosis. Since more thorough experimental investigations into tumor-induced amyloidosis and the accompanying serum-protein changes seen in electrophoresis have not been published, we feel justified in reporting our findings in this paper. Preliminary communications have appeared recently (*13–15*).

MATERIALS AND METHODS

Our materials and methods were described in detail in a previous publication (*16*). Urines were procured by gentle pressure on the bladder through the abdominal wall and were collected in capillary glass tubes. The mice were (CBA × DBA/2)F_1 hybrids, preferably females. The paper electrophoreses were run on 10 μl. of serum at 100 volts for 16 to 17 hours, with an acetate-barbital buffer, *p*H 8.6, ionic strength 0.05. The immunoelectrophoretic technique was a somewhat modified form of Scheidegger's micromethod (*17*).

The spontaneous neoplasm used was a reticulum-cell sarcoma originating in a 31-month-old untreated (CBA × DBA/2)F_1 female mouse from an experimental series of mice previously described (*18*). An autopsy revealed that the left kidney had been replaced by a whitish, fleshy tumor mass 10 × 10 × 10 mm.; the right kidney was partly infiltrated and the peripheral lymph nodes were slightly enlarged. The liver and spleen and other organs were normal in appearance. Under microscopic examination the tumor was found to be a syncytial pyroninophilic reticulum-cell sarcoma; the spleen, right kidney, and lymph nodes were infiltrated by reticulosarcomatous cells. No signs of amyloidosis could be detected. Further description of the tumor cells will be given, together with the findings in the mice that received grafts.

Incidence of Takes and Survival Time

Minced tumor tissue was injected subcutaneously into 6 (CBA × DBA/2)F_1 hybrid mice; the grafts grew in 2 mice that survived for 15 and 25 months, respectively. In the following 9 passages in which 105 mice were used, the incidence of takes was 100 percent, except in the third passage when the grafts grew in only 3 of 5 mice.

The survival time has varied to a great extent within each transfer generation—156 to 260 days in the third passage and 26 to 124 days in the seventh passage.

The Serum-Protein Changes

With few exceptions serum from all mice that received grafts was examined on paper electrophoresis; simultaneously total serum protein

was estimated. Blood for these examinations was drawn just before death of the mice. The development of the protein changes was followed regularly in many mice. The same serums from several mice have been investigated by immunoelectrophoresis. Concurrently urinary-protein excretion was followed by paper electrophoresis and selected specimens of urine were examined immunoelectrophoretically.

Serum-Protein Changes Demonstrated by Paper Electrophoresis

The changes in serum protein are shown in figure 1. The first change from normal appearance observed in the mice with grafts was a more or less marked hypergammaglobulinemia found coincidently with the proliferative, pyroninophilic phase of the splenic and hepatic reticular cells (*vide infra*); next an increase of the β-fraction began and later the α_2-fraction increased; the hypergammaglobulinemia eventually disappeared.

When the serological changes had reached this stage of development, most mice died, and microscopic examinations revealed widespread tumor infiltration, particularly in the spleen, liver, and lymph nodes, with varying degrees of amyloidosis mainly in the spleen and liver.

In mice whose tumor growth took a more protracted course, the changes just mentioned developed further; the albumin and the β-globulin fractions gradually faded, whereas the α_2-fraction steadily increased (fig. 1). In extreme cases, half the protein in the serum was located in the α_2-fraction when these mice died. Mice living until this stage had conspicuous renal amyloidosis.

Table 1 shows the development of these paper electrophoretic changes in relation to increasing survival time and pathological changes in organs of the 6 mice of the fifth transfer generation all killed when moribund. Table 2 gives the electrophoretic stages during amyloid development in a single mouse.

An abnormal protein fraction (fig. 1) in the slowest part of the γ-region in front of the application line was observed in several mice just before death, usually in mice with a rapid, extensive tumor growth and consequently with a short survival time; this protein fraction was particularly conspicuous from the seventh transfer. By staining the paper electrophoretic strips for carbohydrate and lipide, it was evident that this fraction contained only minute quantities of these components.

Total serum-protein determinations showed that the level often rose above normal when the β- and α_2-fractions increased. In our normal mice, 6.1 to 6.7 gm. percent serum protein was found but in amyloidotic mice up to 8.5 gm. percent. In mice not succumbing in this stage of tumor development the protein level again dropped to about 5 gm. percent or even less (*see* tables 1 and 2). In mice with rapid tumor growth there was no increase in total protein, but a terminal decrease to about 3.5 to 4 gm. percent was observed.

Urinary excretion of protein.—Mice normally excrete protein in the urine, and with our technique of electrophoresis, the protein fractions migrated with the mobility of albumin and prealbumin.

TABLE 1.—Correlation of the degree of sarcomatous infiltration, amyloid precipitation, serum-protein changes, and morphologic blood changes with the survival time of all 6 mice of the fifth transfer generation of transplantation line 37

	Microscopic lesions											Electrophoretic determination												
	Tumor infiltration*					Amyloid precipitation*						Relative percent					Gram percent						Differential count	
Survival time (*days*)	Local growth	Lymph nodes	Spleen	Liver	Kidney	Local growth	Peripheral lymph nodes	Spleen	Liver	Kidney	Total serum protein (*gm. %*)	Albumin	α_1	α_2	β	γ	Albumin	α_1	α_2	β	γ	Number of white blood cells	Lymphocytes	Granulocytes	Monocytes
39	100	95	85	85	0	0	0	10	5	0	4. 3	17	2	23	34	24	0. 7	0. 1	1. 0	1. 4	0. 5	10200	61	27	12
56	100	95	80	60	0	0	0	10	20	0	5. 8	30	3	23	32	12	1. 7	0. 2	1. 3	1. 9	0. 7	15900	50	41	9
64	100	95	60	30	10	0	0	20	60	20	8. 3	25	5	23	31	16	2. 1	0. 4	1. 9	2. 6	1. 3	35700	36	58	6
86	100	95	0	0	0	0	0	80	70	20	7. 8	32	3	21	26	18	2. 5	0. 3	1. 6	2. 0	1. 4	16600	43	42	15
91	100	95	0	0	—	0	—	90	80	—	5. 7	21	4	38	23	14	1. 2	0. 2	2. 2	1. 3	0. 8	13600	12	86	2
108	100	95	0	0	0	0	0	95	85	70	5. 3	7	1	45	33	14	0. 4	0. 1	2. 4	1. 7	0. 7	14300	8	87	5
						Ten normal mice	Mean				6. 2	59	5	10	13	12	3. 6	0. 3	0. 6	0. 8	0. 7	6713	78	20	2
							Minimum				6. 0	54	3	8	11	9	3. 3	0. 2	0. 5	0. 7	0. 6	3640	75	12	0
							Maximum				6. 6	64	8	15	16	17	4. 0	0. 4	0. 9	1. 0	1. 0	12530	85	24	4

*The figures indicate percent of sarcomatous infiltration and amyloid precipitation, respectively, in the total area of the site named.

TABLE 2.—Development of electrophoretic serum-protein changes and of morphologic blood changes seen in the same mouse of the sixth passage during growth of the reticulum-cell neoplasia in transplantation line 37

Days after inoculation	Days before death	Total serum protein (*gm.* %)	Electrophoretic determinations											Differential count		
			Relative percent					Gram percent					Number of white blood cells			
			Albumin	α_1	α_2	β	γ	Albumin	α_1	α_2	β	γ		Lymphocytes	Granulocytes	Monocytes
114	146	7.3	44	3	14	17	22	3.3	0.2	1.0	1.2	1.6	5300	58	31	11
147	113	7.7	47	4	15	17	17	3.6	0.3	1.2	1.3	1.3	6600	64	31	5
197	63	8.2	42	5	14	23	16	3.4	0.4	1.2	1.9	1.3	6300	54	31	15
218	42	7.7	40	5	20	23	12	3.1	0.4	1.5	1.8	0.9	22300	25	48	37
259	1	5.4	11	2	48	24	15	0.6	0.1	2.6	1.3	0.8	3200	12	74	14
260	0	5.2	11	3	52	21	13	0.6	0.1	2.7	1.1	0.7	3600	5	91	4

Abnormal urinary excretion in such high concentrations that it could be observed in electrophoresis of unconcentrated urine was only seen in mice with a definite renal amyloidosis. In such mice electrophoresis of the urine showed a gradually increasing excretion of the β-globulin in addition to the normal elimination of protein, whereas the excretion of the greatly enhanced α_2-fraction of serum was minimal. Figure 2 shows electrophoreses (each one run on 20 μl. of unconcentrated urine) of 5 consecutive stages of the urinary-protein elimination, all from the same mouse.

No excretion of the slowly migrating, abnormal protein fraction was observed.

Serum-Protein Changes Observed in Immunoelectrophoresis

Immunoelectrophoretic findings in serum from mice in the early stages of the sarcomatous and amyloid development as compared to normal mouse serum, the polyvalent antiserum 20 HV being used, are shown in figure 3.

The precipitation bows of normal mouse serum have been thoroughly described in previous papers (*17*, *19*). The findings due to casein- and sarcoma-induced amyloidosis have been reported recently (*15*) and are more completely illustrated than in the present paper. The immunoelectrophoretic method does not allow for more than semiquantitative estimations; its power of resolution, however, is higher than that of other methods, as 21 different proteins have been demonstrated in normal mouse serum (*loc. cit.*)

Figure 3 shows that the γ-bow is closer to the antibody reservoir and much more extended in the anode-cathode direction than in the normal serum. This change is the result of an excess of γ-antigens, consisting of the $\beta_{3\text{-II}}$-, $\beta_{3\text{-III}}$-, and the γ-globulins, with the bows of the former 2 proteins usually superimposed on the anodal part of the γ-bow (*19*, *20*), and thus indicates a definite hypergammaglobulinemia. In addition in some serums not shown in figure 3, a precipitation bow larger than normal, probably $\beta_{2\text{-IV}}$, was seen. The bow was located in the β_1-β_2-area medially to the γ-bow and anodally starting near the central filling hole.

Figure 3 shows that the mobility and possibly the concentration of the $\beta_{2\text{-III}}$-bow has increased. The latter change was found more and more pronounced as the tumor growth increased. However, the changes in the $\beta_{2\text{-III}}$ are not specific for this particular sarcoma, but have been found during growth of all transplanted and spontaneous neoplasms examined and after the parenteral administration of foreign protein (*15*, *16*, *21*, *22*). The role of this protein and the reason for its changing, under the circumstances mentioned, are unknown at present.

In addition to the changes in the γ- and $\beta_{2\text{-III}}$-lines, an increased concentration of the double-curved $\beta_{3\text{-I}}$ precipitation bow, particularly of its cathodal part, is also seen in figure 3; this change, however, was not seen as constantly as other changes.

Figure 4 shows the changes in serum from mice with a fully developed splenic and hepatic amyloidosis with the increase in the β- and, particularly, α_2-fractions in paper electrophoresis (fig. 1). Here immunoelectrophoresis revealed a higher mobility and a more or less markedly increased concentration of the $\alpha_{2\text{-I}}$- and $\alpha_{2\text{-II}}$-proteins; of the latter changes the increased mobility of the $\alpha_{2\text{-I}}$-fraction was most conspicuous and most regularly observed.

When a definite renal amyloidosis had developed, the findings were complicated by an increase in the $\alpha_{2\text{-V}}$-globulin and less regularly in the $\alpha_{2\text{-III}}$, whose precipitation lines were seen much more extended in the anode-cathode direction than normally (fig. 5). With further development of the renal amyloidosis other changes became conspicuous, especially a decrease in the albumin and γ-globulin level, as estimated from their less-marked precipitation bow located at a greater distance from the antibody reservoir.

These changes were seen simultaneously with the high level in the α_2-fraction and the reduction in the albumin and β-globulin, in paper electrophoresis (fig. 1), in the terminal stage of the disease. Thus the huge α_2-fraction here corresponds to a combined increase in the $\alpha_{2\text{-I}}$-, $_{2\text{-II}}$-, $_{2\text{-III}}$-, and $_{2\text{-V}}$-fractions in immunoelectrophoresis.

Serums, which in paper electrophoresis showed the slowly migrating fraction just mentioned (fig. 1), in immunoelectrophoresis revealed stages of the described changes except those that were protracted, which were seen in some cases. Often $\beta_{2\text{-IV}}$ could be seen more or less distinctly (fig. 31), but the characteristic finding was changes in the γ-bow. In some cases a sudden deviation of the γ-bow toward the antibody reservoir revealed a localized excess of antigens of γ-globulin nature either in the β-1-area concentric around the central filling hole (fig. 30) or in the slow γ-area. The latter deviation preferably seen in mice of the latest passages was of the same type as observed in paraproteinemia in transplantable plasma-cell leukemias (*21*), although of more cathodal localization. Simultaneous deviations in both areas have also been found. The paraproteinemia was associated with hypo- normo-, or hypergammaglobulinemia of the nonaffected γ-areas. In other cases (fig. 31), the deviation was but slightly pronounced or not present at all; the γ-bow, however, was located nearer the antibody reservoir and reached further nodally and cathodally than normally indicating a diffuse increase of all parts of the γ-bow. Immunoelectrophoresis left the impression that the abnormal protein fraction seen in paper electrophoresis represented a paraprotein-like γ-globulin, whose mobility was very slow as is evident particularly from the paper electrophoreses.[3]

[3] In immunoelectrophoresis the localization of the deviation in the β-1-area around the central filling hole is most likely explained by hampered migration of the paraprotein resulting from aggregation or poor solubility owing to the low ionic strength of the agar medium combined with a cryoglobulin or marked euglobulin character of the protein. Deaggregation or increased solubility may account for the increased mobility of the paraprotein to its true localization in the slow γ-area. Similar changes in mobility, probably caused by enzymatic hydrolysis, have been observed in some cases of human myeloma (J. Heremans: Les globulines sériques du système gamma. Editions Arscia, S.A., Brussels 1960, pp. 175–177). Slightly different pore size in various preparations of agar gel may also have influenced the hampering of the migration.

Ultracentrifugation experiments were performed,[4] at 60,000 rpm in an oil-driven ultracentrifuge (Svedberg type), on serums diluted 4 times in phosphate buffer *p*H 6.66, containing 0.2 M sodium chloride. Serums from a normal mouse and from 3 mice showing the abnormal γ-fraction were investigated. In the normal serum, 3 components were seen with sedimentation coefficients 4.5, 7.8, and 17.9S, comprising 89, 2, and 9 percent, respectively. In the 3 pathological serums a considerable increase of the globulin component was the main change: 38 percent of a 6.8S component was present in one serum, 25 percent of a 7.2S component in the other and 27 percent of a 6.9S component in the third. The light component was similar to that of normal serum, though of smaller percentage; the heavy component constituted the same percentage as in normal serum. In one abnormal serum the sedimentation coefficient, however, was 16.2S, in the other a 19S component constituted 3 percent, a 22.8S component 7 percent, and in the third serum a 17S component constituted 10 percent.

Immunoelectrophoresis of the urine from mice which had been shown by paper electrophoresis to eliminate protein in large quantities (fig. 2) revealed the presence of most of the proteins found in the serum; particularly large amounts of albumin and $\beta_{2\text{-I}}$, corresponding to transferrin in human serum, were present.

Blood Changes

Total and differential white blood cell counts (*16*) were made routinely when blood was taken for electrophoretic analyses.

Tables 1 and 2 show that there was a steadily increasing, relative, and often absolute, granulocytosis. A varying number of the granulocytes were more or less immature, with ring-shaped nuclei. Furthermore numerous apparently normal monocytes and less normal-looking monocytoid cells were found (figs. 6 and 7). Similar blood changes were seen in mice bearing a transplantable plasma-cell leukemia irrespective of the presence or absence of malignant plasma cells (*16*). In the mice of the second transfer generation a conspicuous number (up to 20%) of large cells were observed which were similar to reticulum cells, yet had a slight plasma-cell resemblance (figs. 8 and 9). They may have been precursors of the monocytoid cell, but more likely were tumor cells (*vide infra*). In the later transfers, however, when the tumor cells were seen in smears of ascitic fluid (figs. 10 and 11) or in imprint preparations (fig. 16), they were much more like definite plasma cells. A few cells of the same appearance were observed in the blood smears from some mice; in a single mouse of the seventh transfer these cells comprised 65 percent of 33,100 white cells.

Macroscopic Lesions

All mice receiving grafts were autopsied. At the site of inoculation, subcutaneously in the left flank, a local growth was found varying from

[4] Through the courtesy of Dr. Robert Djurtoft, Research Laboratories, Carlsberg Breweries, Copenhagen.

a few mm. in diameter to a widespread tumor mass covering the whole flank. The tumors were rather firm; their cut surface was gray and moist and necrotic areas were often seen in the center.

All peripheral, abdominal, and retroperitoneal lymph nodes were more or less enlarged (up to 12 mm. in diameter), particularly those of the axillary and inguinal areas adjoining the graft; the latter often reached a size surpassing the graft. The capsules were stretched but not ruptured and occasionally the thymus was enlarged.

Usually the liver and spleen had increased in size, often to an enormous extent, were lighter in color than normal, and of a fragile, crisp (diffusely sarcomatous), or more paste-like (amyloid) consistency; and in addition circumscribed solid tumor nodules, 2 to 12 mm. in diameter, might be be seen in the parenchyma. In a few mice, ascites (up to 2–3 ml.) developed.

The extensive degree of amyloidosis which characterizes this transplantation line was evident, by gross examination, from the typical pale color and the paste-like consistency of the hepatic and splenic parenchyma. These organs might be found completely converted into amyloid masses, or might be the seat of diffuse sarcomatous growth exclusively, or a mixture of these changes might be found in the same organs.

The kidneys occasionally were infiltrated by sarcomatous growth from the superior renal lymph nodes, but more often appeared shrunken and had a pale yellowish-brown color, with their surface granular and blurred indicating heavy amyloid development.

The sarcomatous infiltration of the abdominal cavity was characterized by the predominance either of extensive lesions of the hepatosplenic type or of a retroperitoneal lymphatic type of tumor growth.

Microscopic Lesions

With a few exceptions the local grafts, lymph nodes, lung, liver, spleen, kidney, and other selected organs from all mice were examined. Besides fixation in 4 percent formalin, fixation in 96 percent ethyl alcohol was used in selected cases. All specimens were stained with hematoxylin-eosine, van Gieson, Unna-Pappenheim, and periodic acid-Schiff (PAS). In addition, methyl violet, Giemsa, toluidine blue, alcian blue, and congo red were used, and impregnation methods for reticulin and metalophilic reticulum cells (*23*).

Sarcomatous Changes

The local grafts and the spontaneous tumor (fig. 12) were composed of infiltrating reticulum cells (fig. 13), in various degree of organization; the cells were seen as syncytial masses or isolated with distinct round outlines. The nuclei, generally located centrally, were elongated, with scanty chromatin, distinct nuclear membranes, and most often one or more pyronine-positive nucleoli; the abundant cytoplasm was slightly basophilic and pyroninophilic (fig. 14). Several mitoses were observed.

and in many areas there was a tendency toward fibroblastic or plasmoblastic differentiation (fig. 15). Various degrees of infiltrative power of the tumor cells into the surrounding tissues were noted. Reticulin fibrils were scarce (fig. 17) and seemed to be closer to metalophilic and slightly PAS-positive reticulum cells of normal morphology than to the nonmetalophilic tumor cells (fig. 18). The invaded tissue spaces always contained many mast cells.

In Giemsa-stained smears of ascitic fluid the tumor cells appeared as free, isolated cells of varying size (figs. 10 and 11); the usually centrally located nuclei were round or slightly indented with a delicate but intensely staining chromatin network containing 1 to 3 nucleoli; a brim of moderately basophilic somewhat cloudy cytoplasm surrounded the nuclei. Numerous mitotic cells and a few cells with 2 nuclei were observed. In imprint preparations of tumor tissue or of infiltrated organs (fig. 16) the tumor cells were of the same appearance, though not suspended in fluid and often seen with contiguous cell borders.

In Giemsa-stained preparations the tumor cells thus assumed an unmistakable resemblance to immature plasma cells, indicating that the tumor cells, although of reticulosarcomatous appearance in sections, are differentiated in the plasma-cell direction.

In mice of the second transfer generation that had reticulum-cell-like cells in the blood, the tumor cells had a more histiocytic, more polymorphous appearance, with pleomorphic nuclei and larger although varying chromatin content, than that observed in the other passages. Multinucleated giant cells were present. In the smaller vessels and the sinuses of the liver, numerous tumor cells were seen.

In the group of mice mentioned which had a rapid extensive tumor growth, no amyloidosis, and the abnormal, slowly moving serum-protein fraction, the dominant cell of the local growths (fig. 32) was a large reticulum cell with distinct cell borders and of polygonal form; an abundant, slightly pyroninophilic cytoplasm surrounded the round, centrally located nucleus which had a distinct nuclear membrane, scanty chromatin, and usually a nucleolus. Other diffusely scattered cells or clusters of cells were seen with less abundant but more pyroninophilic cytoplasm and nuclei resembling those of young plasmocytes; there were nearly mature plasmacellular elements with a clear perinuclear space next to an eccentrically situated nucleus (figs. 32 and 33). In the spleen the reticulum cell predominated, while in the liver the predominant neoplastic cells were the transitional forms of plasmoblastic character. Thus these growths exhibited a more pronounced plasmocytoma-like differentiation than the reticulum-cell sarcomas in the mice with amyloidosis. Transitional stages between these tumors and the "ordinary" reticulum-cell sarcomas were observed.

Lymph nodes.—Usually the capsule was preserved but stretched and surrounded with and/or infiltrated by cells that had a plasma-cell appearance (fig. 19). Such cells also occurred in large numbers in the marginal sinus and in the medullary cords. Cells resembling those seen in the

grafts filled the rest of the nodes, with germinal centers and lymphocytes disappearing, so that no real landmarks of the original structure remained. With rapid development of the disease, the invading cells were more the histiocytic type, with evidence of strong infiltrating power; thus the capsule was completely destroyed.

Liver.—Solid tumor nodules of varying size, diffuse infiltration of the sinuses, and perivascular and endovascular tumor proliferations were seen (fig. 22). In extreme cases these changes were so pronounced that almost no liver tissue was left. The tumor cells had less abundant but more pyroninophilic cytoplasm and rather dark and polymorphous nuclei (fig. 20).

Spleen.—Tumor nodules composed of large polygoneal cells with abundant pyroninophilic cytoplasm developed in the follicles, gradually overgrowing them (fig. 21). Simultaneously tumor cells of polymorphous appearance were seen in the perifollicular zone and in the red pulp. In most extreme cases the malignant change caused complete obliteration of the normal splenic architecture.

Other organs: ovaries, uterus, adrenals, pancreas, intestine, and stomach.)—Infiltration of rather polymorphous but still pyroninophilic reticulum cells were most often seen, although syncytial proliferations of identical cells might be found.

The observed sarcomatous changes conform with those of the reticulum-cell neoplasm, Type A, described by *Dunn* (*24*).

Amyloid Development

The organs most frequently and markedly the seat of amyloidotic changes were the spleen, liver and kidney, in the order mentioned, but amyloid formation was observed in many other organs.

Spleen.—The amyloid formation started in the perifollicular zone (fig. 21), where the conversion from a proliferative pyroninophilic phase to a PAS-positive amyloid secreting phase of the reticular cells was observed in some animals. In later stages increasing areas of amyloid precipitations were formed until in extreme cases very small remnants of splenic tissue remained (fig. 23).

Liver.—All grades of amyloidosis up to almost complete displacement of the hepatic tissue by amyloid precipitations were seen (figs. 22 and 24). In a few cases the initial cellular changes in amyloid formation were observed, *viz.*, hypertrophy and pyroninophilia of Kupffer cells succeeded by conversion to cytoplasmic PAS-positivity, and then to secretion of amyloid material.

Kidney.—The principal amyloid lesion was conversion of the glomerular tufts to amyloid masses, and in addition, the interstitial reticular tissue between the renal tubules was often amyloid-affected (fig. 25), these lesions being associated with cystic distension of the tubules which contained casts, as first described by Dunn (*1*).

Lymph nodes.—The PAS-positive reticulosis usually seen in the marginal and medullary sinuses showed conversion into amyloid precipitations,

especially in the pancreatic lymph nodes (fig. 26). Amyloid development was also found occasionally in the mesenteric lymph nodes, but was rarely seen in the peripheral. In the lymph nodes with no tumor infiltration a severe plasmocytosis was often observed (fig. 29).

Other organs.—In several other organs, amyloid transformation was found most often in the pancreas, stomach, intestine, adrenals (fig. 28), and ovaries (fig. 27). In the pancreas, it was only located in the walls of the vessels; in the ovaries the amyloidosis could not be distinguished from involution by age, but the material reacted tinctorially like amyloid.

No amyloid development was observed in the heart, tongue, cutis, or in the grafted tumors.

Tinctorial properties of the amyloid.—The amyloid material stained positive with PAS and alcian blue, negative with toluidine blue and methyl violet, but it reacted metachromatically to toluidine blue after fixation in alkaline lead-acetate. It was only weakly stained by congo red; yet this dye gave the amyloid a pink fluorescence when examined by the fluorescence microscope. As recently reported (*25*) this method was particularly valuable for the detection of minor amounts of amyloid.

Coexistence of sarcomatous and amyloidotic changes.—All degrees of alternating amyloidosis and tumor growth were observed within the same organs; some areas were amyloid, while others were sarcomatous, but small amyloid precipitations and single tumors cells were also seen side by side.

In mice with a short survival time, in addition to a smaller or larger local growth, extensive sarcomatous infiltrations into distant organs were usually seen, whereas there was only a slight formation of amyloid in the organs. With increasing survival time, the degree of amyloidosis in the spleen and liver increased, and the areas of sarcomatous infiltration in these organs were less extensive until, in mice with a protracted course of the disease, the spleen and liver were found heavily amyloidotic but with little or no sarcomatous infiltration (figs. 23 to 25). The renal amyloidosis developed later than the splenic and hepatic one.

Table 1 shows the microscopic changes seen in all 6 mice of the fifth transfer generation arranged according to increasing survival time. Table 1 also shows the correlation of the serum-protein changes, with the progression of the pathologic changes in the organs.

Correlation of Electrophoretic and Morphologic Findings

The earliest changes observed were a hypergammaglobulinemia, manifesting itself in immunoelectrophoresis as an increase in the $\beta_{3\text{-II}}$-$\beta_{3\text{-III}}$-γ-globulin group, which probably corresponds to the $\beta_{2\text{-A}}$ system in human serum; often an increase in $\beta_{2\text{-IV}}$, which probably corresponds to $\beta_{2\text{-M}}$, a human, macromolecular, antibody-carrying globulin, was also seen. The correspondence of the latter proteins is very likely, as judged from the fact that $\beta_{2\text{-M}}$ is seen highly increased in Waldenström's macroglobulinemia in man (*26*), and a supposed $\beta_{2\text{-IV}}$-bow has been found

strongly increased in mice bearing a transplantable leukemia, exhibiting the same histologic changes as are characteristic of human macroglobulinemia (*27*).

Since the proteins that had increased presumably belong to the antibody-containing globulins, the so-called immunoglobulins (*28*), the early stage of the serum-protein changes, which coincided with the proliferative, pyroninophilic phase of the splenic and hepatic reticuloendothelial cells, may be regarded as representing a stage of hyperimmunoglobulinemia.

The $\beta_{3\text{-I}}$-protein which could be seen increased in this stage probably corresponds to $\beta_{\text{I-A}}$-$\beta_{\text{I-C}}$ of human serum, possessing some activity like the third part of the complement (*29*). It is rather tempting to consider the increase in this protein also as representing a mobilization of the immune mechanism.

As mentioned, the constantly observed increase in the $\beta_{2\text{-III}}$-bow is a reaction seen so far with all types of tumor growth of unknown pathophysiologic significance. The increased β-fraction observed in paper electrophoresis probably represents the $\beta_{2\text{-III}}$.

Simultaneously with the formation of amyloid in the spleen and liver, an increase in mobility and more or less markedly in concentration of $\alpha_{2\text{-I}}$ (corresponding to human α_1-glycoprotein) and of $\alpha_{2\text{-II}}$ (probably corresponding to human α_2-haptoglobin) was observed, these changes in paper electrophoresis manifesting themselves as an increased α_2-fraction.

The last stage of the serum-protein changes, which were characterized in paper electrophoresis by a definite reduction of the albumin and β-globulin fractions and by a rise of the α_2-fraction, and in immunoelectrophoresis by an increase in the $\alpha_{2\text{-V}}$- and sometimes the $\alpha_{2\text{-III}}$-globulin developed, while progressing renal amyloidosis caused a lesion of the glomerular membrane and thus gave rise to the development of a nephrotic syndrome. The decrease in serum albumin and β-globulin and the simultaneously observed renal loss of these proteins, identified preferably as albumin and $\beta_{2\text{-I}}$-globulin (transferrin in man) in immunoelectrophoresis no doubt are due to this syndrome.

In man the relative large elimination of these proteins is explained by their low molecular weights, this elimination resulting in a higher relative concentration of high-molecular proteins as α_2-lipoprotein and Schultze's α_2-macroglobulin (*30*) in serum (*31*).

The human α_2-lipoprotein corresponds to the $\alpha_{2\text{-III}}$-lipoprotein in mouse serum and $\alpha_{2\text{-V}}$-globulin is supposed to be the equivalent of α_2-macroglobulin in man. The nephrotic syndrome thus has given rise to similar protein changes in man and the mouse. Moreover, it has to be stressed that the serum-protein changes developing in this last stage of amyloidosis, with the kidneys affected, must be explained as a consequence of the nephrotic syndrome and only indirectly owing to the amyloidosis.

As will appear from the preceding, the serum-protein changes observed during amyloid development were unspecific in the sense that they

consisted in a modification of the amount of, and the ratio between, the normally occurring protein fractions; no evidence of the production of abnormal protein fractions due to amyloid development was observed.

In some mice, especially those with a short survival time, with a slightly developed amyloidosis, and with a more plasma-cell differentiated microscopic picture of the tumor growths, immunoelectrophoresis revealed a change in the γ-protein of a type similar to that seen in paraproteinemic plasma-cell neoplasias. The findings in ultracentrifugation are also in agreement with an excess of γ-globulin. The paraprotein-like nature of this abnormal protein makes it most likely that it is produced by the tumor cells, whether by the reticulum cells or by the more plasma-cell differentiated elements or both is unknown. In this connection it should be mentioned that administration of cortisone to mice grafted with tumor caused an enhanced appearance of the abnormal γ-fraction in serum and a further plasma-cell differentiation of the growths (unpublished experiments).

Whether the slightly increased β_{2-IV}-fraction partly or wholly is a product of the tumor cells, as it has been shown to be in another transplantation line (*27*), is undecided so far. Further experiments into the problems associated with the development of the abnormal serum proteins are currently in progress.

DISCUSSION

The present line of transplantation has been named a reticulosarcomatosis, based on the multilocated spreading of the tumor growth and on the reticulum-cell-like appearance of the tumor cells in sections. However the cytoplasm of most cells was strongly pyroninophilic; and isolated tumor cells in the marginal sinuses of the lymph nodes and in smears and imprints clearly resembled immature plasma cells. Therefore, a certain plasma-cell differentiation of the tumor cells has to be assumed. Whether this differentiation has been of any importance to the ability of the tumor cells to induce amyloidosis is unknown. We have observed 4 other lines of reticulosarcomatosis, all of the Type A described by Dunn (*24*), and numerous lines of plasma-cell leukemia, with and without the presence of abnormal serum-protein fractions (*18*), while amyloidosis was only seen sporadically in these lines.

For years amyloidosis has been reported to occur in mice with spontaneous or transplanted tumors. The amyloidosis observed in the present line, however, was definitely more marked and more frequent than with other tumors previously reported. In fact, amyloidosis developed so regularly that it has been practicable to use this tumor in investigations (to be published) into the acceleration of amyloid formation.

The morphology and the morphogenesis of the sarcoma-induced amyloidosis were similar to our observations in casein-induced amyloidosis (*13*, and unpublished experiments) and to those of others (*4*, *5*, *8–12*, *32*), thus indicating that sarcoma- and casein-induced amyloidosis

develop through a common pathogenesis. Moreover, the morphology of secondary amyloidosis in mice has been found identical to that in other species, including man (*8, 9*).

Many hypotheses of the pathogenesis of amyloidosis have been advanced. Our morphologic findings fit naturally with Teilum's theory (*7–9, 33*) in which the emphasis is on the assumption that amyloid is produced as a local secretion from the reticuloendothelial cells and does not consist of precipitates derived from the circulating blood. Amyloidosis is developed as a response to a persistent stimulation of the immune mechanism through a first stage of active proliferation of the reticuloendothelial cells, pyroninophilia indicating an enhanced production of ribonucleic acid, into a second phase in which PAS-positive granules are seen in the cells. According to Teilum the PAS-positive granules are evidence of the production of polysaccharide containing proteins. If the antigenic stimulus continues, a dysfunctional phase develops, perhaps because of exhaustion of the protein synthesis of these cells, presumably provoking a disturbance in the action of an enzyme system, with the consequence that an abnormal secretion product is deposited as amyloid material around the cells, eventually causing suppression and obliteration of the cells.

Our electrophoretic studies, which have revealed identical protein changes during the development of casein- and sarcoma-induced amyloidosis (*14, 15*), agree with those of others (*4, 10, 32, 34*), with the older techniques used in many of the experiments taken into consideration. When correlated to the simultaneously occurring developmental stages of the amyloid changes in the organs, our serologic findings also conveniently may be explained in accordance with the view of Teilum.

The nondevelopment of amyloidosis in mice killed by rapid extensive tumor growth might be thought to be due to the reduction in the number of the host's amyloid-forming reticular cells, as they are crowded out by the tumor growth, and to the insufficient length of time left for the formation of amyloid. Moreover, since it is known that γ-myeloma proteins show no action as antibodies in the humoral defense mechanism in man (*35*), it is conceivable that the nondevelopment of amyloidosis may also be due to the paraprotein-like nature of the γ-protein in these mice, such pathologic proteins being unable to participate unlike normal γ-globulins in the immunization, the primary, etiological factor in amyloid development. But why some mice and not others develop this pathologic protein fraction and the associated characteristic histologic tissue changes is unknown at present; experiments into this problem are currently in progress.

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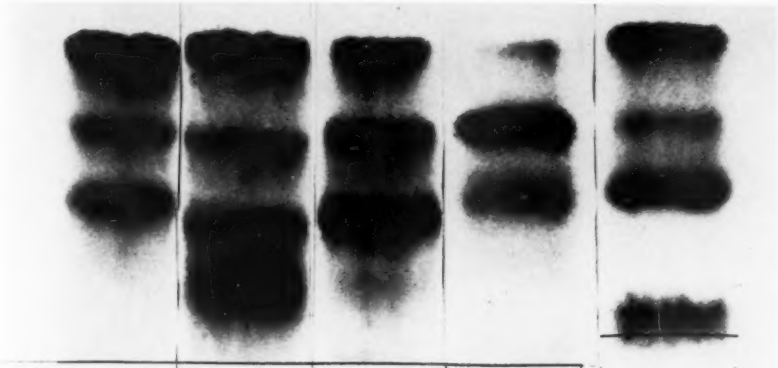
PLATE 27

FIGURE 1.—Paper electrophoreses of serum from mice of third passage with progressing development of sarcoma-induced amyloidosis. *Strip left:* normal serum. *Second strip* from *left:* first phase of serum-protein changes, *e.g.*, hypergammaglobulinemia. *Third strip,* from *left:* next phase—marked increase of β- and α_2-globulin fractions. *Fourth strip* from *left:* last (nephrotic) phase consisting in reduction of the albumin and β-globulin fractions and distinct increase of α_2-fraction. *Strip* to *right:* pathologic, very slowly migrating protein fraction, seen in some sarcoma-bearing mice (*see* text); simultaneously amyloidosis-induced increase of β-globulin fraction visible. All strips run on 10 μl. of serum.

FIGURE 2.—Paper electrophoreses of urine from mouse of sixth passage during development of sarcoma-induced amyloidosis; the urine collected during last 66 days of 128-day survival time. *Strip left:* physiological excretion of protein, which on *next strip* to *right* is seen to be distinctly reduced; on following *three strips* the renal loss particularly of albumin and β-globulin is illustrated. Strips were run on 20 μl. of unconcentrated urine; amount of total protein and date of excretion indicated on each strip.



	%	gm %	%	gm %	%	gm %	%	gm %	%	gm %
alb.	60	3.5	34	2.4	22	1.7	8	0.4	25	1.3
α_1	3	0.2	4	0.3	2	0.2	1	0.1	7	0.4
α_2	11	0.6	15	1.1	27	2.1	37	2.0	14	0.8
β	12	0.7	18	1.3	34	2.6	31	1.7	22	1.2
γ	14	0.8	29	2.0	15	1.1	23	1.3	8	0.4
									24	1.3
total protein		5.8		7.1		7.7		5.5		5.4

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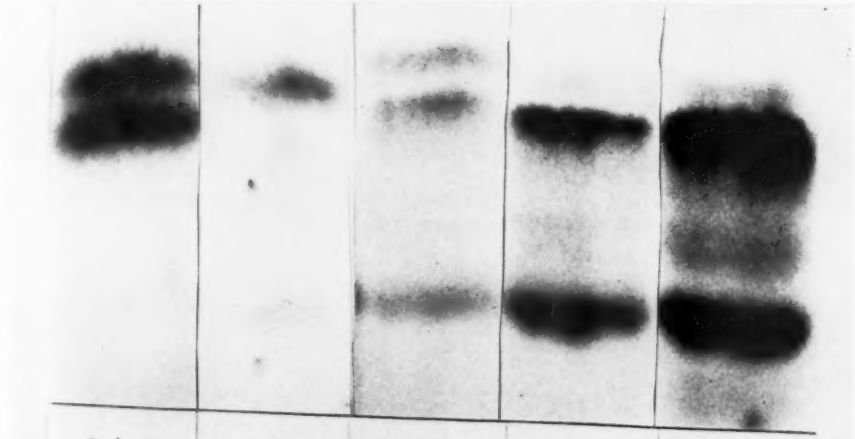

10/2-59	20/3-59	23/3-59	3/4-59	13/4-59
1.7 g % prot.	0.4 g % prot.	1.0 g % prot.	1.5 g % prot.	2.3 g % prot.

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PLATE 28

Immunoelectrophoresis of serum from mice with progressing development of sarcoma-induced amyloidosis.

FIGURE 3.—A: Serum from mouse in early stage of amyloid development. By comparison with normal serum run on the same slide (B) the following changes are seen: the system of the $\beta_{2\text{-II}}$-$\beta_{2\text{-III}}$-γ-bows is of increased extension and located nearer the antibody reservoir indicating hypergammaglobulinemia. The cathodal bow of the double-bent $\beta_{2\text{-I}}$-fraction, although faintly represented, extends more cathodally than in normal serum and is seen to cross the γ-bow, which is not true in normal serum. The $\beta_{2\text{-III}}$-bow is of increased extension anodally; changes in this bow, however, are seen with all types of malignant growth and are thus independent of the amyloid development. B: normal serum run together with A.

FIGURE 4.—C: Serum from mouse with manifest splenic and hepatic amyloidosis, diluted 1:4. Besides the changes seen in figure 3 an increased mobility of the $\alpha_{2\text{-I}}$- and $\alpha_{2\text{-II}}$-bows is visible. D: normal serum run together with C, diluted 1:4.

FIGURE 5.—E: Serum from normal mouse, diluted 1:4, and run together with F: serum from a mouse with marked renal amyloidosis, diluted 1:4. A heavy concentration of the $\alpha_{2\text{-V}}$-bow is evident by comparison with diluted normal serum, in which the $\alpha_{2\text{-V}}$ is seen as a very weak precipitation line. The reduced concentration of albumin and γ-globulin in the pathologic serum may also be observed.

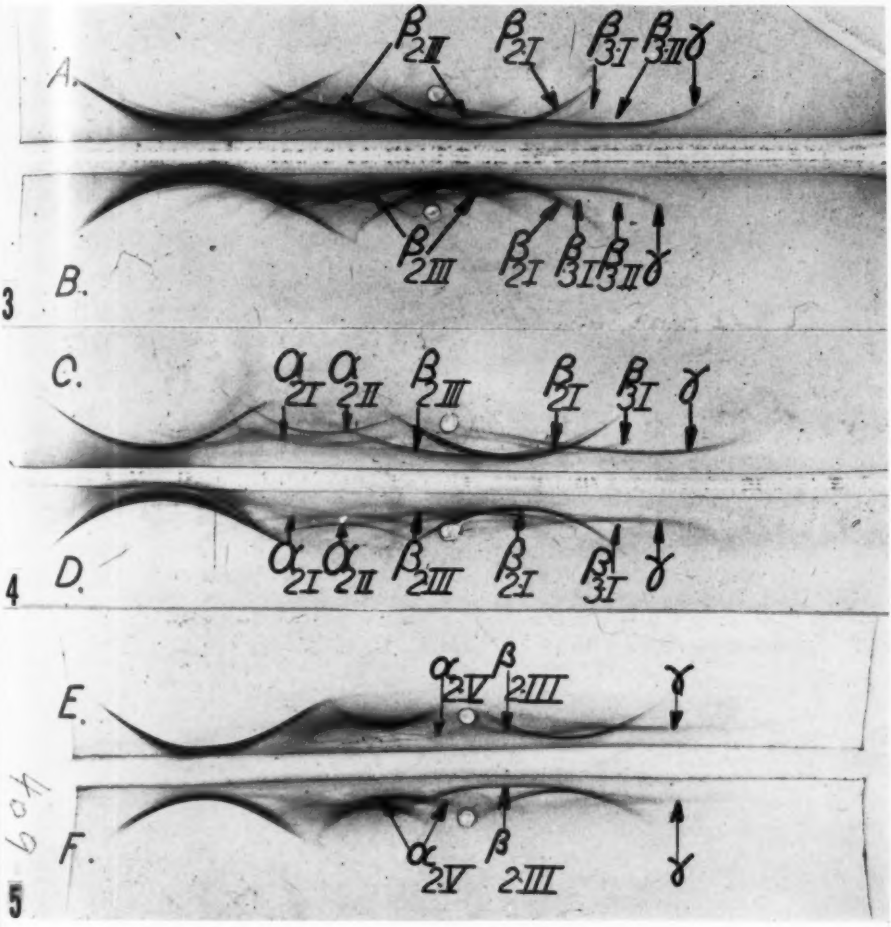

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PLATE 29

FIGURE 6.—Smear of blood. Monocyte with round nucleus, moderate chromatin content, with slightly basophilic cytoplasm. Giemsa stain. × 1,800

FIGURE 7.—Smear of blood. Monocytoid cell with indented nucleus, coarse chromatin structure, and slightly basophilic cytoplasm. Giemsa stain. × 1,800

FIGURES 8 and 9.—Smear of blood. Large reticulum-cell-like cells with large nuclei containing large nucleoli and abundant, slightly basophilic cytoplasm. Giemsa stain. × 1,800

FIGURE 10.—Smear of ascitic fluid. Tumor cells with large round nuclei, delicate chromatin pattern, and 1 or more nucleoli. Scarce cytoplasm basophilic, cloudy, and vacuolized. *Below:* binucleate cell like that of dividing plasma cells. Giemsa stain. × 1,800

FIGURE 11.—Smear of ascitic fluid. Large reticulum cell *below* has monstrous nucleus, with segmentation. *Above right:* a plasmoblast of malignant type. Giemsa stain. × 1,800

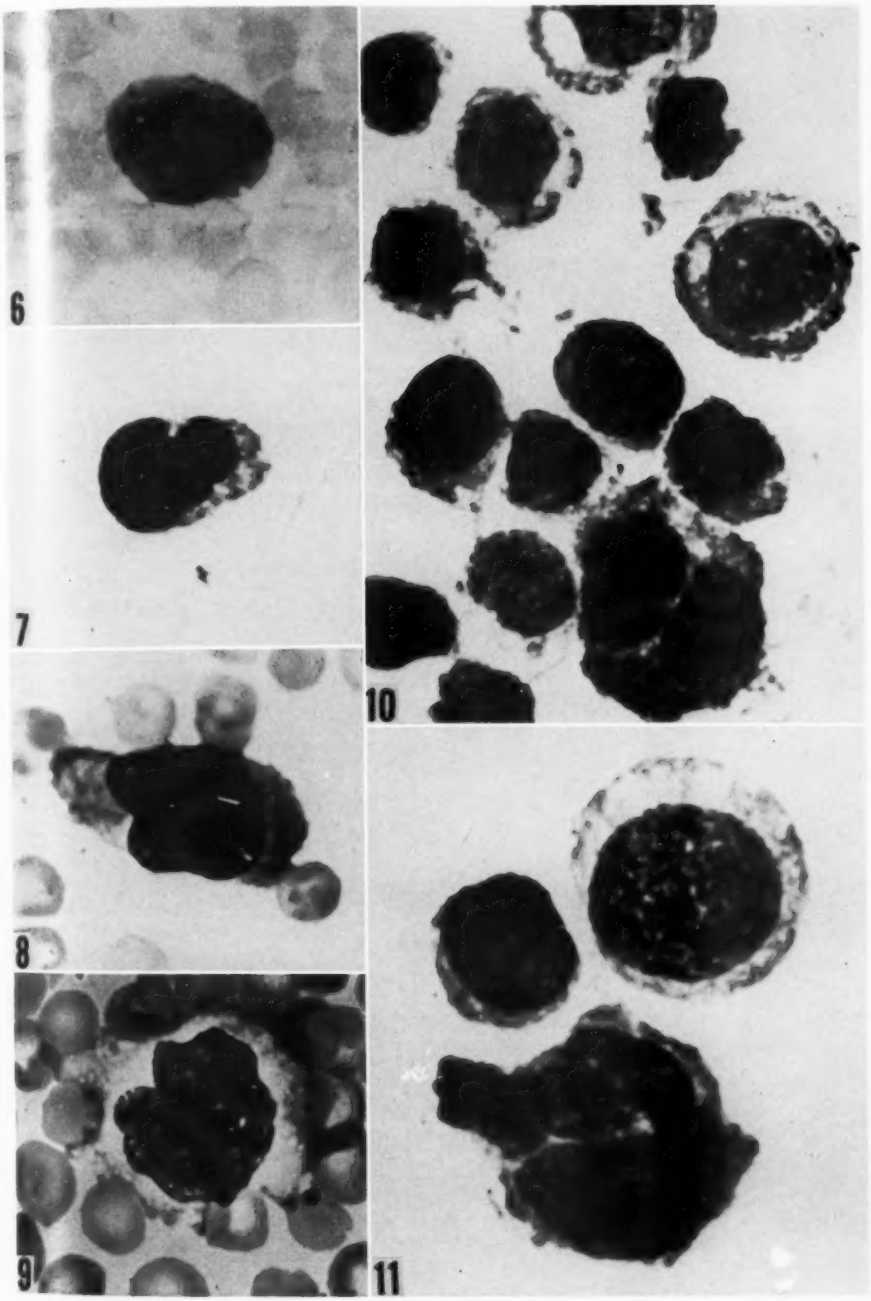
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PLATE 30

FIGURE 12.—Local graft. Shows tumor growth invading underlying striated musculature from subcutis. Invaded tissue spaces always contain many mast cells. Hematoxylin and eosin. × 75

FIGURE 13.—High-power view of syncytial area in figure 14. Reticulum cells have round or oval nuclei poor in chromatin content, often with pyroninophilic nucleoli; cell borders are inconspicuous; cytoplasm contains only sparse pyroninophilic granules. Unna-Pappenheim. × 1,450

FIGURE 14.—Local growth. Tumor composed partly of syncytial masses of pale reticulum cells and partly of histiocytic cells, the latter with pyroninophilic cytoplasm. Unna-Pappenheim after ethanol fixation. × 490

FIGURE 15.—High-power view of histiocytic area in figure 13. Nuclear membrane has become stronger; chromatin structure not so loose and nucleoli have diminished in size when compared to cells in figure 14. This change in appearance of tumor cells characterized by presence of free cells most of which have developed pyroninophilic cytoplasm and sometimes perinuclear halo has been interpreted as differentiation phenomenon. Unna-Pappenheim. × 1,450

FIGURE 16.—Imprint preparation of local graft. Several tumor cells showing different stages of development. Most have large round nuclei with moderate chromatin content and slightly basophilic cytoplasm. In *lower* part of figure nuclei are smaller and contain more chromatin. Giemsa stain. × 1,450

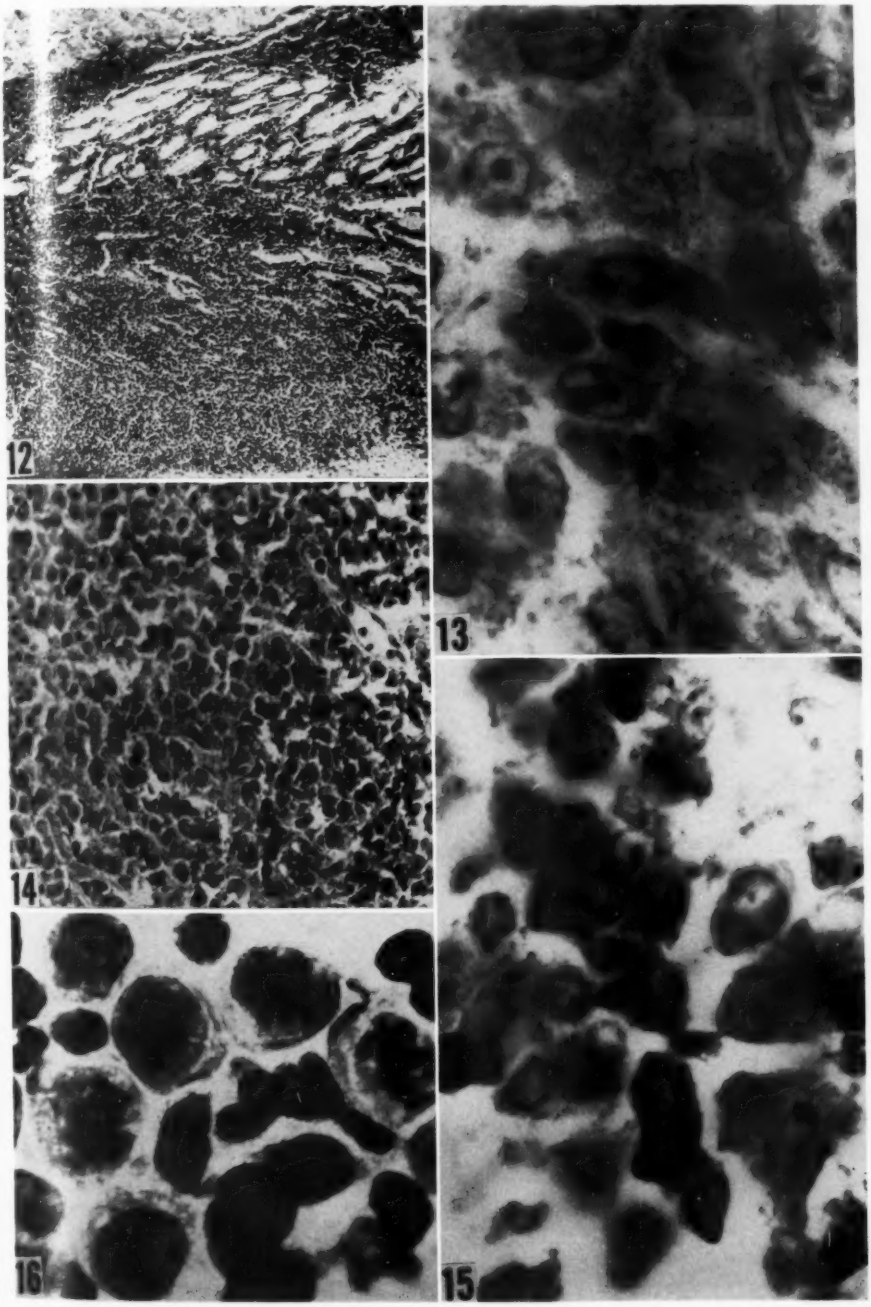
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PLATE 31

FIGURE 17.—Spontaneous tumor. Reticulin fibrils seem to follow connective-tissue components, vessels, and capillaries of tumor; not be taken as sign of real reticulin production formed by malignant cells. Reticulin stain by Wilder's method. × 280

FIGURE 18.—Spontaneous tumor. Number of metalophilic reticulum cells rather abundant—could not be stated with certainty that some tumor cells were metalophilic. Impregnated cells are probably supporting cells, between which the tumor cells lie in lacunae, pericytes, and mesenchymal cells of invaded tissue. Spontaneous tumor morphologically showed a fibroblastic deviation. Metalophilic reticulum cells stained by Marshall's method. × 280

FIGURE 19.—Tumor-infiltrated lymph node. Whole node overgrown by reticulosarcomatous cells, but where tissue spaces are left, there is tendency to plasmoblastic differentiation of malignant type. Capsule invaded by such cells and also the perinodal and perihilar regions. Hematoxylin and eosin. × 145

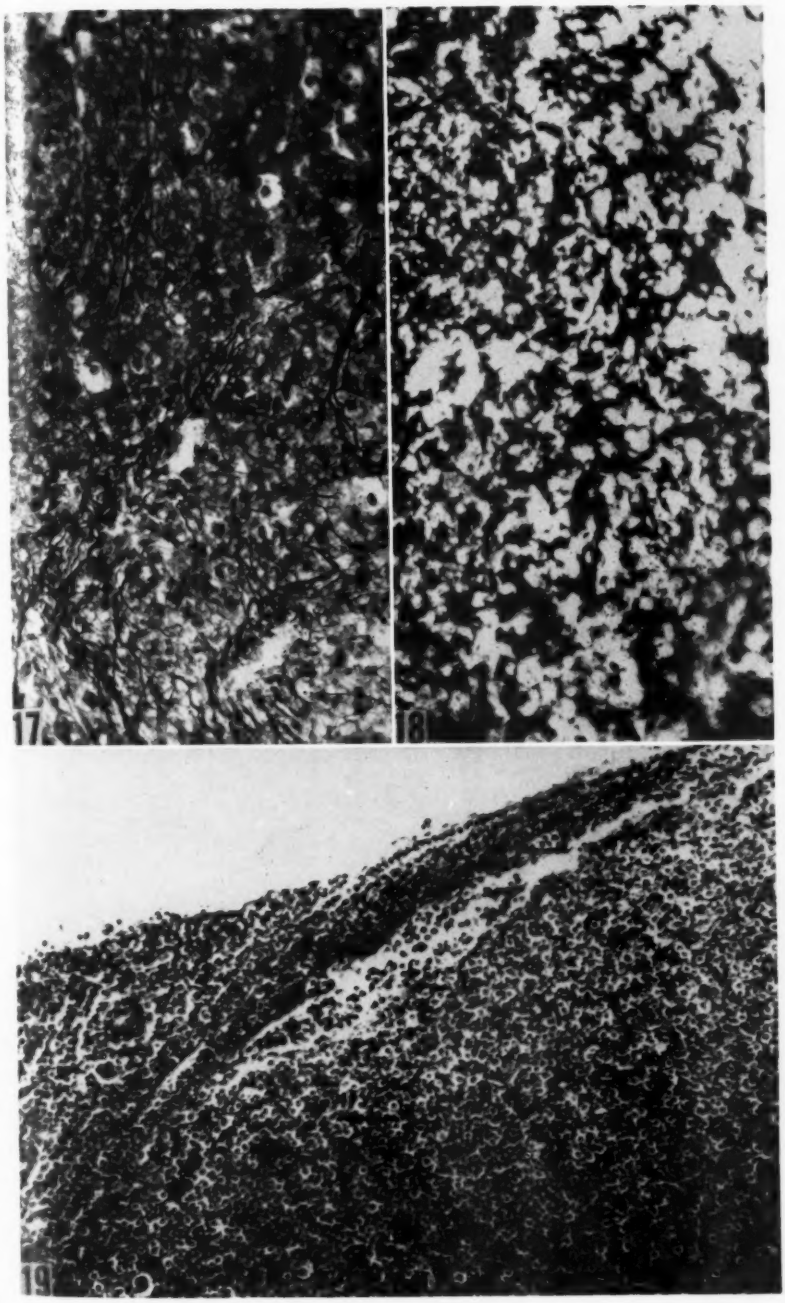
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PLATE 32

FIGURE 20.—Tumor growth in liver. Tumor cells show polymorphic picture. Some cells have hyperchromatic nuclei and pyronine-positive cytoplasm; others are malignant reticulum-cell type. Localization of the chromatin in bits just beneath nuclear membrane is typical for other cells. Unna-Pappenheim. × 1,050

FIGURE 21.—Section of spleen showing coexistence of sarcomatosis and amyloidosis. Splenic follicle enlarged owing to malignant reticulum-cell proliferation. Cells of perifollicular collar compressed, while those of perifollicular halo have formed amyloid material. Red pulp shows increased extramedullary hematopoiesis. Unna-Pappenheim. × 145

FIGURE 22.—Section of liver illustrating coexistence of tumor growth and amyloid formation. Only few liver cells remain as compressed streaks. In *upper* half to *left* perivascular tumor-cell proliferation around a small amyloid-changed vessel is seen. Amyloid deposits occlude most sinuses. Some amyloid-producing cells may be seen dimly in *lower* half of figure. PAS technique. × 490

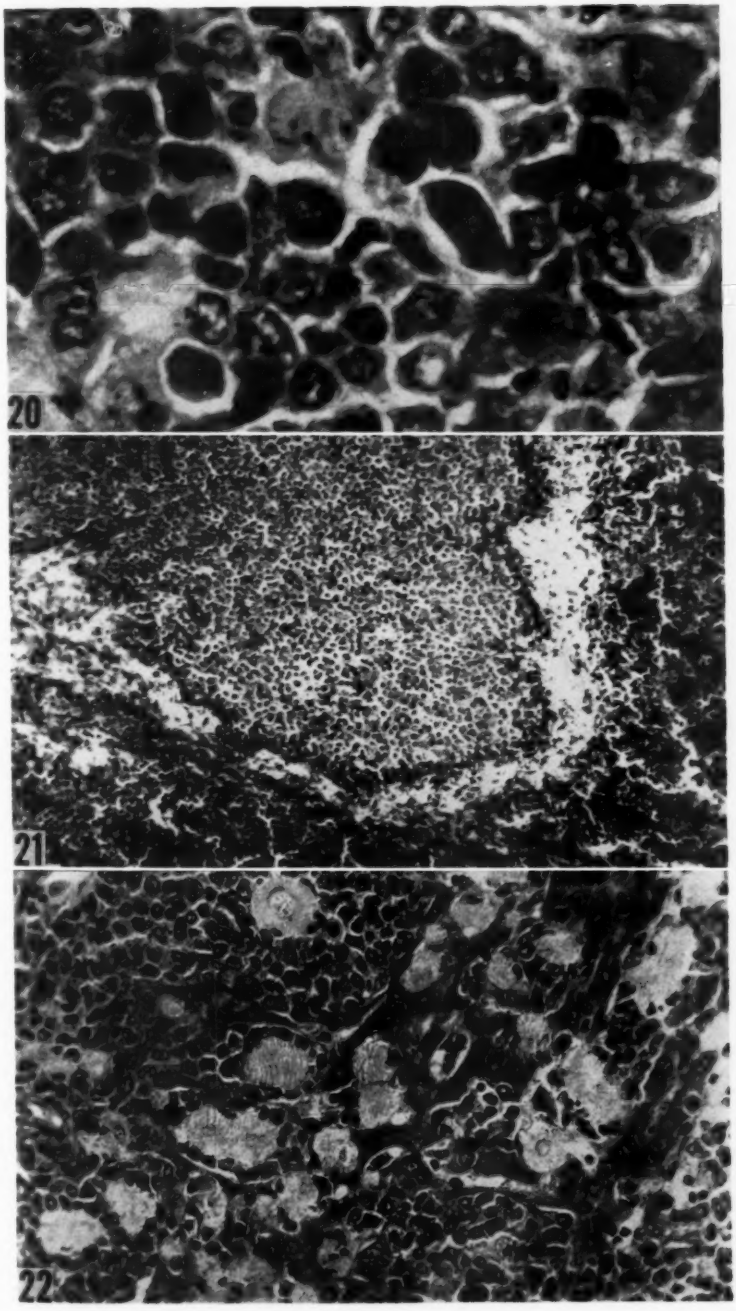
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PLATE 33

FIGURE 23.—Section of amyloid-transformed spleen. Splenic structure totally destroyed by formation of amyloid, which is not so amorphous as supposed. Fine fibrillary appearance distinguishable especially around vascular structures where the color also is most intense. PAS technique. × 75

FIGURE 24.—Section of amyloid-transformed liver. Only venae centrales, some compressed liver-cell cords, and sinuses remain of original structure. Remainder of organ replaced by amyloid deposits showing same staining intensities as splenic amyloid material. PAS technique. × 75

FIGURE 25.—Section of amyloidotic kidney. Characteristics of the amyloid disease of mouse kidney are all present. All glomeruli amyloid-changed, tubules distended, and some tubules filled with a PAS-positive material; interstitially amyloid formation also seen but to minor degree. PAS technique. × 75

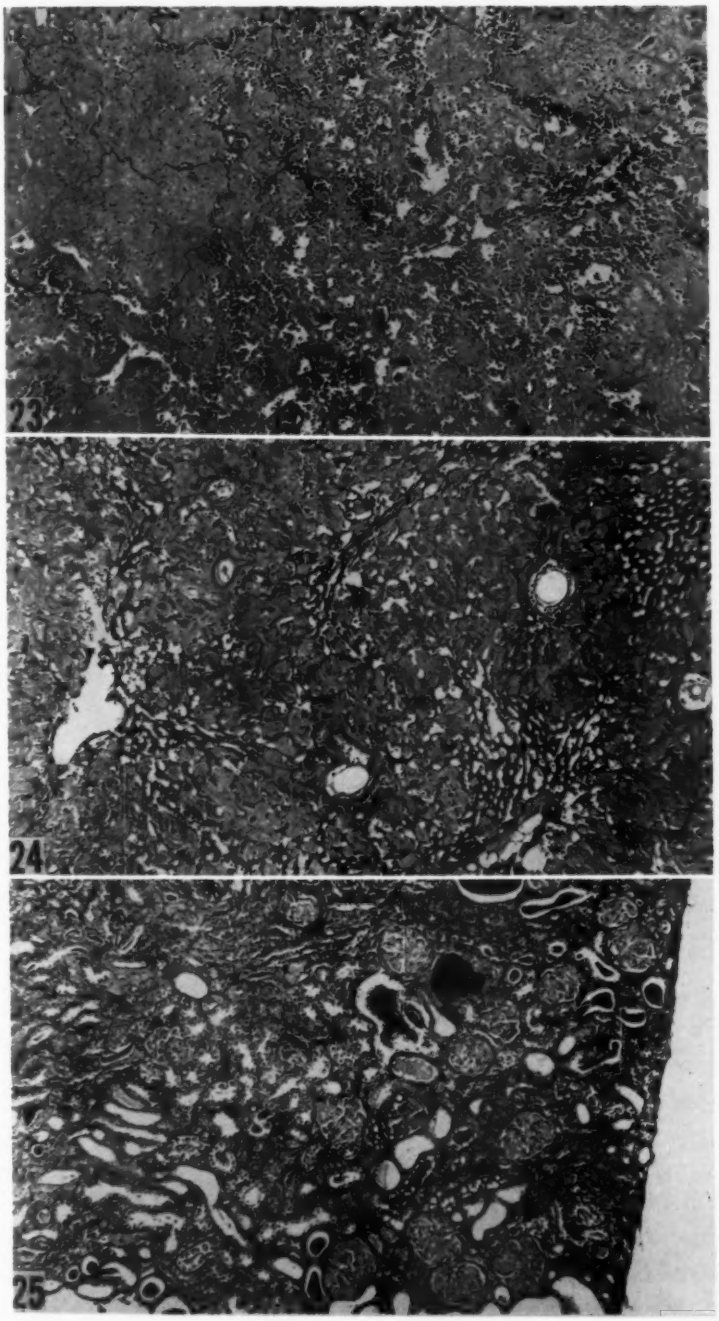
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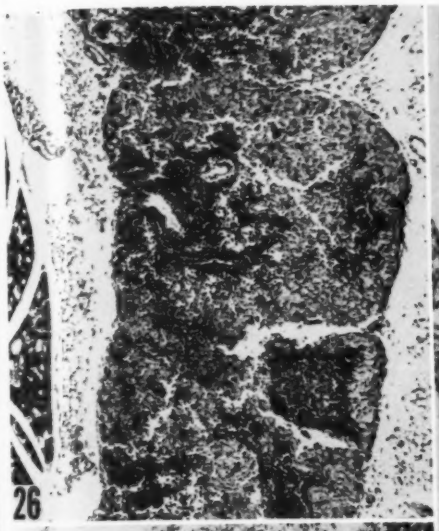
PLATE 34

FIGURE 26.—Amyloid development in pancreatic lymph node. Structure of lymph node only partially preserved; amyloid deposits occupying sinuses. PAS technique. × 75

FIGURE 27.—Amyloid development in ovary, from an old mouse, but the atretic corpora lutea have same morphology and staining reactions as seen in amyloid deposits. Hematoxylin and eosin. × 75

FIGURE 28.—Amyloid formation seen in zona reticularis of the tangentially cut adrenal gland. PAS technique. × 75

FIGURE 29.—Imprint preparation of lymph node showing severe plasmocytosis often observed in lymph nodes in areas without tumor infiltration. Giemsa stain. × 1,450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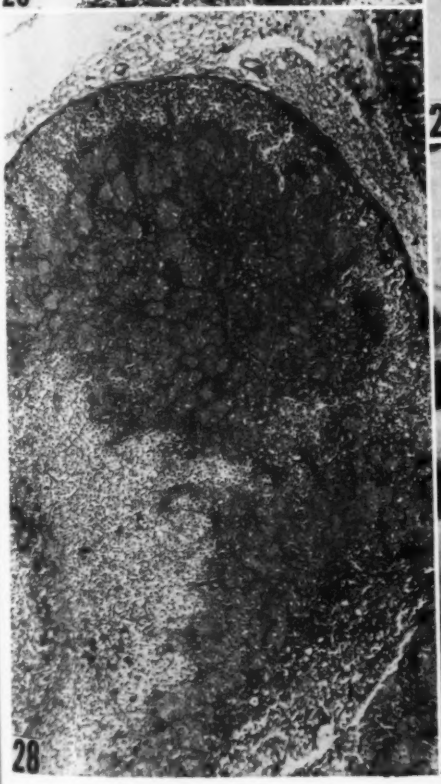
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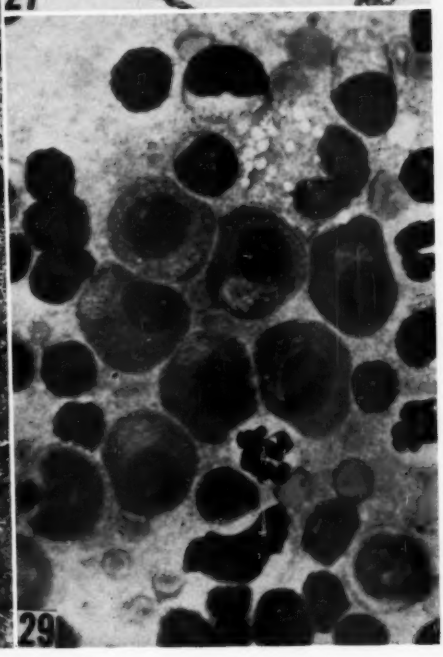
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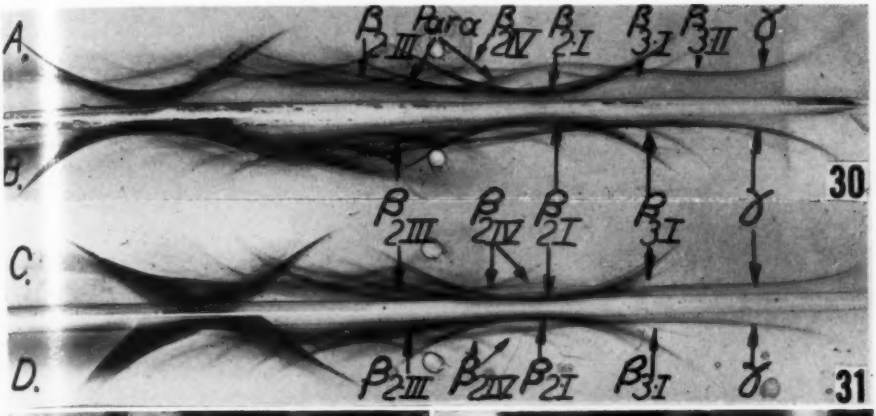
PLATE 35

FIGURE 30.—A: Immunoelectrophoresis of serum in paper electrophoresis showing abnormal, slowly migrating globulin fraction. In anodal end of γ-bow a sudden deviation, named Para, toward the antibody reservoir indicates excess of abnormal protein in this area; the cathodal part of γ-bow is normal in shape but of shorter extension and farther from the antibody reservoir than normally demonstrated hypogammaglobulinemia in slow γ-region. $\beta_{2\text{-IV}}$ seems only slightly increased. B: Normal serum run together with A.

FIGURE 31.—C: Immunoelectrophoresis of serum showing less marked changes in γ-bow than serum seen in A. Only slight deflection of fast part of γ-bow is seen, which extends farther anodally than normally, indicating a more diffuse increase in fast part of γ-bow. Cathodal end of γ-bow is nearer antibody reservoir and longer than normally revealing hypergammaglobulinemia in this region. $\beta_{2\text{-IV}}$ is of larger extension than in normal serum and in serum shown in A. D: Normal serum run together with C

FIGURE 32.—Local growth from mouse with slowly migrating, abnormal serum protein fraction. Tumor tissue is composed of sarcomatous reticulum cells intermingled with numerous atypical plasma cells of varying degree of maturation. A clear, perinuclear space is seen in some of plasma cells. Unna-Pappenheim. $\times$ 1,750

FIGURE 33.—Saline suspension of cells from local growth from mouse with slowly migrating, abnormal serum-protein fraction. Three plasmacellular elements corresponding to those seen in figure 32 are shown. Localization of nucleus and its chromatin structure, typical for plasma cells, is evident. Well-developed granules in abundant cytoplasm surround the nucleus at some distance from it. Phase contrast. $\times$ 2,700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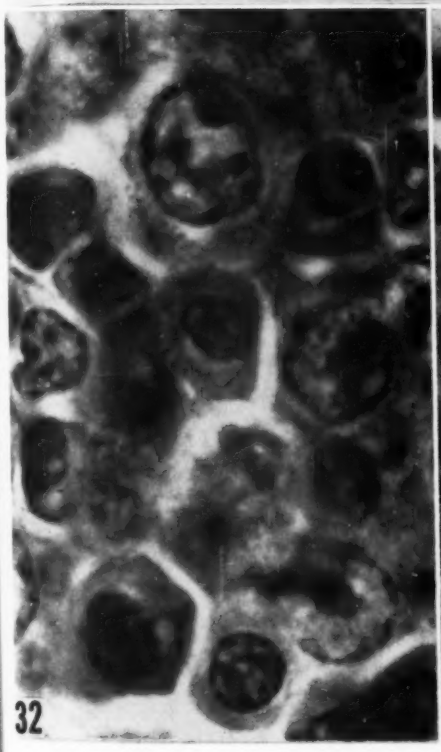
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Genetic Response of Inbred Lines of Chickens to Rous Sarcoma Virus [1]

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SUMMARY

This study demonstrates the genetic variation among families of White Leghorn chickens, representing 5 different and unrelated inbred lines, when they were inoculated intracerebrally at 1 day of age with Rous sarcoma virus. The differences in response to inoculation of chickens with Rous sarcoma virus, both within and between lines, were marked. The progeny from line 10 representing 4 different sire matings, each mated to different groups of females, were completely susceptible and all died from the effects of the disease induced by inoculation. In contrast the progeny from line 7, representing 3 different sire matings, were highly resistant and only 2 chickens of 327 died. Progeny from lines 6, 9, and 15 showed considerable genetic diversity in response to Rous sarcoma virus. Within lines the progeny from certain males showed considerable resistance, while the progeny of other males in these same lines all died before 25 days of age. Likewise, progeny from dams, within sires and lines, showed similar genetic diversity in response to Rous sarcoma virus, some being completely resistant and others completely susceptible.—J. Nat. Cancer Inst. 25: 351–357, 1960.

THE EXPERIMENTAL variation found in chickens in response to inoculation with Rous sarcoma virus (RSV) was reviewed by Bryan (*1*). The present study emphasizes the importance of the genetic constitution of the host chicken when variables associated with RSV preparation and administration are held reasonably constant. Numerous studies have demonstrated genetic differences in resistance to Rous sarcoma (*2–6*). Rarely (*2*, *6*) have highly inbred lines of chickens been available for such genetic studies. Burmester *et al.* (*6*), reporting on the use of mixed multiple-sire-flock matings of chickens from all the inbred lines used in the present study, demonstrated marked line differences in response to both subcutaneous and intracerebral inoculations with RSV, administered at 3 different laboratories.

Data obtained on the response of the chickens to RSV inoculations,

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herein reported, stress the importance of the genetic reaction of progeny from individual sires and dams.

MATERIALS AND METHODS

All the chickens used in this study for response to inoculation with Rous sarcoma virus were White Leghorns and represent 5 different and unrelated inbred lines. These chickens have been maintained at this Laboratory for the past 21 years without the introduction of any outside stocks. During this period the basis for selection and development of these lines of chickens was to obtain differences in resistance and susceptibility to naturally occurring avian lymphomatosis (*7*, *8*). More recently, chickens from the same inbred lines used in this study were exposed to experimentally induced erythroblastosis and they showed marked differences in genetic resistance, as reported by Waters *et al.* (*9*). The coefficient of inbreeding for the chickens in all lines is now in excess of 95 percent.

Within each inbred line, 3 or 4 different pens of females were each mated to a single male. Thus any influence of different sires and dams on their progeny within each inbred line of chickens is measurable. A total of 1,618 chickens was hatched each 2 weeks, from April to September, 1959, and all were inoculated at 1 day of age.

The RSV preparation (CT750) used as a source of inoculating material throughout this study was obtained through the courtesy of Dr. W. Ray Bryan, of the National Cancer Institute. This material consisted of a standard lot of highly purified microsome fraction of a subcutaneous Rous sarcoma tumor (*10*). Dilution of the RSV, whenever necessary, was made in 0.85 percent sodium chloride solution to which was added 2.0 percent inactivated (56° C./½ hour) horse serum.

All chicks were inoculated by injection of the virus into the right hemisphere of the brain. The use of the brain of young chicks for biological studies of RSV reaction was developed by Groupé *et al.* (*11*). Their technique was modified in this study to the extent that chicks were inoculated intracerebrally without anesthesia. Careful manipulation of the needle minimized trauma to the surrounding tissue of the brain and head.

Groupé *et al.* (*11*) and Vigier (*12*) found that the RSV will propagate in the chick brain and cause death in a relatively short period of time. The typical reaction to intracerebral inoculation of RSV in 1-day-old chicks is the presence of hemorrhagic lesions in the meninges of the brain. Pathological studies on these brain lesions have been described by Duran-Reynals (*13*). His studies show that the microscopic appearance of the sarcoma cells in the brain was similar to that of cells from intramuscular and subcutaneous Rous tumors.

The optimum dose to inoculate the chickens used in this study was determined from previous dilution assays conducted at this Laboratory on

susceptible inbred chickens. Here it was demonstrated that when chicks were inoculated intracerebrally at 1 day of age, 100 percent died at −3.6 logs; 100 percent died at −4.6 logs; 93 percent died at −5.6 logs; and 46 percent died at −6.6 logs. All deaths in this assay study occurred before 25 days of age and all dead chicks had typical brain lesions. Accordingly each chicken involved in this study was inoculated intracerebrally at 1 day of age with RSV preparation at a dose of −5.0 logs. Previous experience with this particular source of virus, essentially corroborating the research of Groupé *et al.* (*11*), had demonstrated that brain lesions rarely were visible before 7 days after inoculation and deaths were few after 25 days of age. Thus in this study all chickens dying during the first 7 days after inoculation were excluded from the data and all experiments were terminated at 25 days after chickens were inoculated.

RESULTS

Mortality following intracerebral inoculation with RSV into 1-day-old chickens belonging to 5 different inbred lines is shown in table 1. Because brain lesions of the type observed in this study are not known to occur among noninoculated chickens, it was not thought necessary to maintain controls. Actually, noninoculated chickens were raised from every family reported in the various lines studied without observing a single instance of Rous sarcoma reaction. No special attempt was made to separate inoculated chickens from noninoculated chickens, though none were in direct contact. Recently, however, Burmester *et al.* (*14*) provided evidence showing that when chickens are inoculated either intravenously or subcutaneously with RSV at a dilution of −4.0 logs and placed in direct contact with noninoculated susceptible chickens these contact chickens will develop a high percentage of typical Rous tumors. When the dilution is reduced to a −6.0 logs for inoculated chickens, a few tumors still appear in the direct-contact chickens. Thus, Burmester *et al.* (*14*), for the first time, clearly demonstrated that under certain conditions Rous sarcoma is contagious.

The mortality response to inoculation of chickens in different inbred lines and of progeny from different sires and dams provides good evidence of genetic differences for resistance and susceptibility to RSV. The chickens in line 7 were the most resistant since only 2 of 327 chickens had lesions and these may have been errors in record keeping. Line 10 chickens were completely susceptible, with every chicken dying before 25 days of age. Considerable variability in mortality existed within sire matings for lines 6, 9, and 15. In line 6, for example, the progeny from sires 1, 2, and 4 all died, whereas sire 3 from this same line showed no losses in the progeny when mated to dam 4 and variable progeny mortality when mated to other dams. Likewise in line 15 the progeny from sires 1 and 3 show a substantial number of deaths (72 and 67%), whereas

TABLE 1.—Ratio of chickens dead at 25 days of age, after inoculation with Rous sarcoma virus at 1 day of age, to total number of progeny produced from single sires and dams by inbred lines in 1959

Dams	Line 6 (*dead/total*)				Line 7 (*dead/total*)			Line 9 (*dead/total*)			Line 10 (*dead/total*)				Line 15 (*dead/total*)		
	Sires				Sires			Sires			Sires				Sires		
	1	2	3	4	1	2	3	1	2	3	1	2	3	4	1	2	3
1	20/20	13/13	4/4	10/10	0/13	0/2	0/30	8/8	0/4	4/15	7/7	11/11	13/13	19/19	12/13	0/8	16/18
2	9/9	14/14	4/8	24/24	0/10	0/13	0/10	5/10	2/11	0/3	4/4	10/10	5/5	11/11	14/16	2/20	18/23
3	14/14		5/5	14/14	0/15	0/13	0/12	3/3	0/15	4/14	6/6	5/5	32/32	7/7	16/19	2/17	10/19
4	17/17		0/16	2/2	0/15	2/18	0/22	4/4	1/11	4/13	12/12	7/7	2/2	14/14	9/16	0/12	8/17
5	16/16		6/6	16/16	0/16	0/8	0/26	9/9	0/13	10/14	3/3		9/9	28/28	8/17	2/10	4/11
6	6/6		3/19		0/16	0/15	0/17		1/10	6/16	6/6				11/13	0/13	8/16
7	17/17		2/10		0/22				1/14	4/13	9/9				13/20	1/11	3/4
8	16/16		9/9		0/24				0/10	1/5	6/6				14/20	0/5	17/24
9			1/1		0/10					6/12	2/2				12/13	0/2	19/21
10										7/20	5/5				14/17	1/19	
11										4/13					15/18		
12										1/5							
13										3/15							
Total	115/115	27/27	34/78	66/66	0/141	2/69	0/117	29/34	5/88	54/158	60/60	33/33	61/61	79/79	138/182	8/117	103/153

the progeny from sire 2, in line 15, mated to 10 different dams had only 8 deaths of 117 inoculated chickens.

Following the inoculation of the different lines of chickens with RSV it was planned to line cross-resistant and susceptible chickens in reciprocal matings. Unfortunately, the dams selected for these cross matings ceased production before sufficient progeny could be produced. One series of crosses, between line 6 involving susceptible sire 1 and line 7 involving 8 different dams whose progeny were resistant, produced 54 progeny, table 2. All 54 of these progeny died following inoculation with RSV. The reciprocal matings between the resistant sire 1 in line 7 and 2 line 6 dams from mating 1 produced only 7 chickens and they likewise all died during the experimental period. Despite the paucity of chickens in these reciprocal matings there is a suggestion of genetic segregation. However, conclusive evidence on the mode of inheritance for resistance to Rous sarcoma virus must await more substantial evidence.

TABLE 2.—Ratio of chickens dead at 25 days of age, after inoculation with Rous sarcoma virus at 1 day of age, to total number of progeny produced from a reciprocal cross between lines 6 and 7 in 1959

Dams Line 6, sire 1	Line 7, sire 1 (*dead/total*)	Dams Line 7, sire 1	Line 6, sire 1 (*dead/total*)
1	3/3	2	12/12
7	4/4	3	9/9
		4	4/4
		5	7/7
		6	8/8
		7	4/4
		8	5/5
		9	5/5
Total	7/7		54/54

DISCUSSION AND CONCLUSIONS

The primary objective of this study was to determine the degrees of resistance to the RSV among progeny of different sires and dams existing within highly inbred lines of chickens maintained at this Laboratory. As previously stated, each of the 5 different inbred lines of chickens had a coefficient of inbreeding in excess of 95 percent. Despite this high degree of inbreeding some of the inbred lines showed marked heterogenicity in response to inoculation with RSV within and between the progeny of certain sires and dams.

In the present experiments in which a particular virus preparation and intracerebral inoculation is used, the dose of −5.0 logs ml. of virus may be considered "optimum" for a measure of the genetic response to RSV among the chickens of the inbred lines used. It is most likely that different virus preparations and concentrations would show different host results. Thus a near-optimum lethal dose or dilution would need to be established or susceptible chickens before host responses could be meaningful. It

has been established that mortality in chickens after inoculation with RSV is proportional to the dose administered. A highly concentrated virus preparation will overwhelm and cause a high mortality, even in chickens that proved to be highly resistant to the dose of virus used in this study. Evidence to substantiate this, with chickens from resistant line 7 as an example, is provided by Burmester *et al.* (*6*), wherein an RSV dose of −1 logs produced tumors in all chickens of line 7 inoculated in contrast to a dose of −5 logs which produced few or no tumors. It is of special interest that inbred line 7 chickens were inoculated with RSV in 1950, 1956, 1958, and 1959, and each test has shown them to be highly resistant to RSV.

The thought might arise that the resistance of certain chickens to Rous sarcoma virus inoculation may be due to egg-transmitted antibody. The genetic evidence does not agree with such a hypothesis. For example, when line 6, sire 1, was mated with 8 line-6 dams, they produced 115 progeny all of which died after inoculation, table 1. When this same line 6, sire 1, was mated to 8 line-7 females they produced 54 progeny, all of which died after inoculation, table 2. These same 8 dams from line 7 when mated to a line-7 sire produced 128 progeny and there was no mortality at all, table 1. It would be difficult to reconcile these data with the presence or absence of antibody transmitted through the egg by the dam. It seems most likely that a genetic interpretation of the results in this report is consistent with the facts.

It can be concluded that genetic differences in host response to RSV exist between all the inbred lines of chickens studied. The response of progeny from certain sires and dams demonstrates clearly that even within lines the genetic effect is marked. The mode of inheritance cannot be determined from this study.

Those involved in mouse-tumor studies have long recognized their nearly complete dependency on highly inbred stocks of mice with which to work. Workers using the chicken as a host animal in experimental tumor studies have been greatly handicapped because of the lack of a source of highly inbred stocks. The findings given in this report and those of Greenwood *et al.* (*4*) emphasize the importance of having known inbred chickens in studies involving Rous sarcoma. Waters *et al.* (*9*) have demonstrated the necessity of using known genetic stocks of chickens for experimental work with virus-induced erythroblastosis.

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Immunological and Karyological Criteria for Identification of Cell Lines [1,2]

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SUMMARY

An investigation was made of the possibility of identifying established cell lines in culture, by the following characteristics: (*a*) morphology, (*b*) sensitivity to HeLa and MCN nucleoprotein antiserums, (*c*) mouse transplantation antigen(s) detectable by skin-grafting techniques, (*d*) sensitivity to poliomyelitis virus, and (*e*) chromosomal complement. It has been demonstrated that some characteristics, in particular (*b*), (*c*), and (*e*), are highly indicative of the species of origin of a given cell line. Thus the spectrums of action of HeLa and MCN antiserums seem to be related to the human and murine origin of the susceptible cells, respectively. Transplantation antigens may persist in established lines *in vitro* as evidenced by their ability to sensitize mice against subsequent skin homografts of appropriate genotype. No evidence has been obtained that mice share any important transplantation antigens in common with 4 different mammalian species. Karyological criteria are highly efficient when distinct markers exist or when differential diagnosis is required between species whose diploid idiograms are markedly different. Even though notable variation occurs during establishment of a cell line, the karyological profile, nontelocentric versus telocentric chromosomes, as an indicator of the species of origin remains fairly constant. For cell lines of doubtful origin, identification is most dependable when the various criteria corroborate one another. Eleven cell lines were tested according to these principles. Four cell lines, 3 of stated human origin and 1 of stated rabbit origin, were actually of mouse origin, probably consisting of L cells.—J. Nat. Cancer Inst. 25: 359–385, 1960.

A RELATIVELY stable and apparently uniform cell line from primary tissue, capable of indefinite proliferation *in vitro*, is the outcome of selective forces acting on a cytologically heterogeneous cell population. The progressive changes undergone during the evolution of such a line may affect cellular morphology, biochemistry, chromosomal characteristics, etc., in unpredictable ways, though none of these changes seem specifically required for survival. Furthermore establishment as a defined line may be a technical illusion in that the cell population may still be capable of undergoing further changes under appropriate conditions.

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[2] Aided by grants C-3577 and C-4534 from the National Cancer Institute, National Institutes of Health, Public Health Service, and grants E-68 and E-89-B from the American Cancer Society, Inc.

Because there are now so many cell lines derived from tissues of at least half a dozen different mammals, confusion has resulted, and the problem of devising means of identifying, or at least confirming, the species of origin of a given cell line has become important. A further complication suggested by several investigators (*1*, *2*) is that contamination of one cell line with cells of another—usually a more vigorous line—may sometimes occur despite rigorous precautions.

This paper presents a critical appraisal of the feasibility of achieving positive identification of a cell line by evaluating 5 characteristics: (*a*) morphology, (*b*) sensitivity to nucleoprotein antiserums, (*c*) detectable mouse transplantation antigens, (*d*) sensitivity to poliomyelitis virus, and (*e*) chromosomal complement.

MATERIALS AND METHODS

Cell lines in tissue culture

The cell lines studied, together with the supposed tissues of origin, the sources of supply for the cells studied, and the culture mediums in which the cells were grown, are listed.

HeLa.—Epidermoid carcinoma of human cervix (*3*); Microbiological Associates, Bethesda, Maryland; medium 199 and 5 percent horse serum.

D-227.—Infant foreskin; Dr. J. Leighton (*4*), University of Pittsburgh, Pittsburgh, Pennsylvania; Eagle's in Earle's medium and 20 percent human serum.

Det-504.—Human testicular teratoma (*5*); Dr. C. Stulberg, The Children's Research Center, Detroit, Michigan; Eagle's in Hanks' solution and 16 percent human serum.

McCoy-A.—Synovial fluid of the knee joint of a patient with degenerative arthritis (*6*); Dr. J. A. Sykes, University of Texas, Houston, Texas; medium 199 and 10 percent horse serum.

McCoy-B.—Synovial fluid of the knee joint of a patient with degenerative arthritis (*6*); Dr. L. Hayflick, The Wistar Institute, Philadelphia, Pennsylvania; medium 199 and 10 percent horse serum.

Amnion Ep.L.—Human amnion epithelial-cell line reisolated from a mixed culture (*7*) originally labeled Fernandes (*8*); The Wistar Institute, Philadelphia, Pennsylvania; Eagle's basal medium and 10 percent horse serum.

Amnion Fb.L.—Fibroblast line reisolated as described for *Amnion Ep.L*; The Wistar Institute, Philadelphia, Pennsylvania; Eagle's basal medium and 10 percent horse serum.

ARF.—Areolar tissue of rat (*9*); Dr. G. O. Gey, The Johns Hopkins University, Baltimore, Maryland; medium 199 and 10 percent human serum.

L.—Subcutaneous tissue of a C3H mouse, clone 929 (*10*); The Wistar Institute, Philadelphia, Pennsylvania; medium 199 and 10 percent horse serum.

MCN.—Human leukemic bone marrow (*11*); Dr. G. Henle, The Children's Hospital, Philadelphia, Pennsylvania; medium 199 and 10 percent horse serum.

Wistar 8.—Adult rabbit kidney (*12*); Dr. E. Larson, Women's Medical College, Philadelphia, Pennsylvania; Eagle's basal medium and 5 percent calf serum.

The cell lines were grown on glass in 8 ounce or 1 liter Blake bottles and were harvested by trypsinization. Only a single cell line was maintained under test in the laboratory at any one time, to avoid mistakes.

RESULTS

Morphological Characteristics

The morphological characteristics of these cell lines have been described by their originators (*see* references). No morphological changes were observed in the cells during the period of maintenance in this laboratory; this was not unexpected since all lines except Det-504 had already been propagated *in vitro* for considerable periods. The cells could be broadly classified as "fibroblast" or "epithelial" on the basis of the pattern of cellular organization and the presence or absence of cytoplasmic processes under the methods of culture used. HeLa and Amnion Ep.L, were epithelial; all the others were fibroblast. Within the latter group some cell lines, such as MCN, L, Wistar 8, Amnion Fb.L, and McCoy-B, were similar in appearance, falling under the L-like prototype. The other fibroblast cell lines, ARF, D-227, and McCoy-A, could easily be differentiated. Of particular interest was line Det-504 which, although passaged more than 15 times *in vitro*, still exhibited morphological features associated with primary fibroblasts.

Cytotoxicity Test

Cells in suspension or on a coverglass were tested for susceptibility to the cytotoxic action of rabbit antiserums (in the presence of guinea-pig complement [3]) which had been prepared against the nucleoprotein fractions of HeLa and MCN cells, by techniques described elsewhere (*13*, *14*). The toxic effect was measured in terms of unstained cell counts, with eosin as the indicator. Exposure of cells to normal rabbit serum at dilution 1:4 furnished the controls.

Although HeLa, D-227, Amnion Ep.L, and Det-504 cells were entirely destroyed by HeLa antiserum at dilutions up to 1:32, they were completely unaffected by MCN antiserum. MCN, L, McCoy-B, Wistar 8, and Amnion Fb.L were destroyed by the MCN antiserum at dilutions 1:16 and 1:32, but were resistant to HeLa antiserum. No cytotoxic activity was observed in normal rabbit serum. ARF cells were not affected by either type of antiserum at dilution 1:4, and only occasional increases in granularity of their cytoplasm were observed. In addition, other tests than those already reported (*14*) were carried out with the HeLa and MCN nucleoprotein antiserums. Their hemagglutinating and hemolytic activity were tested against C3H mouse red cells and type O, A, and B human red cells. The results are reported in table 1. Thus HeLa antiserum agglutinated human red cells of type O, A, and B but MCN antiserum did not; no difference could be demonstrated with mouse red cells because agglutination at the same dilution was present with normal rabbit serum. Hemolytic tests gave results which allowed a further differentiation between HeLa and MCN antiserums (table 2).

[3] Vacsal Complement Lyophilized, Carworth Laboratories, Inc., New York, N.Y.

TABLE 1.—Agglutination*

Serum	Human red cells			Mouse (C3H) red cells
	O	A	B	
Normal rabbit	None	None	None	1:64
HeLa DNP	1:64	1:64	1:128	1:64
MCN RNP	None	None	None	1:64

*Ratios express dilution of serum at which cells are agglutinated.

TABLE 2.—Hemolysis*

Serum	Human red cells		Mouse red cells
	O	A	
Normal rabbit	None	None	None
HeLa DNP	1:12	1:12	None
MCN RNP	None	None	1:48

*Ratios express dilution of serum at which cells are hemolyzed.

To clarify the relationship between hemagglutinating and cytotoxic activities, 0.5 ml. of HeLa DNP antiserum was absorbed with 0.30 ml. of packed human red cells of type O and B for 2 hours at 37° C. and for 24 hours at 4° C. In this way, hemagglutinating activity toward O and B red cells was absorbed with either cell type. When the same antiserum samples were again tested for cytotoxicity against HeLa cells the cytotoxic titer was unchanged. It thus appears that in our antiserums cytotoxic antibodies are not related to blood-group antigens or to other antigens of the red-cell surface.

Sensitivity to Poliomyelitis Virus

The relationship between cellular morphology and sensitivity to certain viruses such as poliomyelitis has been established for several different cell-virus combinations (*15*, *16*). Growth of poliovirus *in vitro* in other cells than those of primate origin is still questionable though by no means excluded. All the cell lines were tested by infection with 0.1 cc. of type 1 Mahoney strain which had an original titer of $10^{7.0}$ TCD50 per ml. in primary monkey kidney cells. On the 7th day, cytopathic effects of this virus were observed only on HeLa, Amnion Ep.L, and Det-504 cells at dilution titers of $10^{7.2}$, $10^{5.4}$, and $10^{5.0}$, respectively.

Test for Presence of Murine Transplantation Antigens

If adult mice are grafted with living tissues, or are inoculated with suspensions of cells of *homologous* origin, they become specifically sensitized to those genetically determined tissue-transplantation antigens in the donor cells which they themselves do not possess. As a consequence of this sensitization the transplanted cells are destroyed (*17*). Whereas skin homografts transplanted to "virgin" or unsensitized hosts normally heal and enter a phase of proliferative hyperplasia before they are rejected, grafts transplanted to specifically sensitized hosts, *i.e.*, animals previously exposed to living cells of the same genetic origin as the skin

homograft, are rejected in a summary manner. They fail to heal properly, their epithelium remains mitotically indolent, and their survival time is demonstrably curtailed. This is the well-known "second-set" phenomenon (*18*).

In tests to be described, adult mice of the selected inbred strain A were inoculated intraperitoneally with an approximately constant, high dose of 6 to 12 million living cells whose species of origin was in question. After 8 days, which gives foreign cells ample opportunity to sensitize their hosts, the latter were challenged with orthotopic skin homografts from a donor of a different inbred strain. Curtailed survival of the test grafts of inoculated animals, as compared with those on a series of uninoculated controls, indicated the sharing of at least one important transplantation isoantigen between the cells and the donor strain of the test grafts, and consequently of the murine origin of the line under test.

In all tests, adult strain A mice of both sexes were used as recipients, and C3H mice provided the skin homografts. This particular combination of strains was selected because (*a*) the median survival time of skin homografts on untreated and on specifically immunized hosts, respectively, differs by 3 or 4 days, so that differentiation between the two types of breakdown, "nonimmune" and "immune," presented no difficulty, and (*b*) it was known that established lines of cells of alleged strain C3H origin are in common use in many laboratories.

The preparation of the donor skin grafts, the operative procedures, and the method of scoring the survival of the grafts have been fully described elsewhere (*19*). Each graft was transplanted to the lateral thoracic wall of the recipient and its survival time estimated on the basis of its outward appearance. The dressings were removed and primary inspections of the grafts carried out on the 6th postoperative day; subsequent inspections were carried out daily.

The survival time of C3H skin homografts of 12 previously untreated strain A hosts and the survival time of similar grafts on 12 hosts inoculated 8 days before with 6 to 12 million C3H spleen cells are shown in table 3. Whereas all the grafts on the untreated mice lived at least 8 days and had a median survival time of about 10 days, none of those on the previously inoculated animals lived longer than 7 days. From the outward appearance of those grafts which manifested the immune type of breakdown it is quite certain that the majority was completely necrotic on the 6th day.

To obtain further evidence for the generally held belief that transplantation antigens are not shared in common between members of *different* species and to provide controls for the tests to be described, panels of strain A mice were injected with freshly prepared cell suspensions from the marrow or spleens of two inbred strains of rat, with spleen cells from rabbit, and with leukocytes of man and rhesus monkey, respectively. The survival time of the C3H test skin homografts borne by the recipients of these different types of cell of heterologous origin (table 3) does not differ from that of the control series and therefore provides

TABLE 3.—Tests for presence of strain C3H mouse tissue-transplantation antigens in cells of various established lines

	Type or line of cell injected	Origin of inoculated cells	Number of strain A mice inoculated*	Survival time of C3H test grafts (*days*)			
				≤7	8–9	10–11	12†
Cells prepared from normal tissues—controls	None	—	12	—	6	4	2
	Spleen	C3H mouse	12	12			
	Spleen	B.N. rat	8	—	2	6	
	Marrow	Lewis rat	5	—	1	4	
	Spleen	Rabbit	7	—	5	1	1
	Leukocytes	Human	5	—	3	2	
	Leukocytes	Rhesus monkey	6	—	3	2	1
Cells from established lines	HeLa	Human‡	6	—	2	3	1
	D-227	Human‡	5	—	—	4	1
	D-504	Human‡	5	—	1	3	4
	McCoy-A	Human‡	6	—	1	5	
	Amnion Ep.L	Human‡	6	—	5	1	
	ARF	Rat‡	6	—	5		
	L	Mouse‡	8	8			
	MCN	Human‡	6	6			
	Wistar 8	Rabbit‡	4	4			
	McCoy-B	Human‡	8	8			
	Amnion Fb.L	Human‡	5	5			

*Each strain A mouse received 6 to 12 million cells intraperitoneally.
†None of these grafts lived longer than 12 days.
‡Alleged.

evidence in support of our initial assumption that the cellular antigens of the class under investigation are highly species specific.

The results of the tests on cells from the 11 lines under investigation are shown in table 3. These cells fall into two distinct classes: (1) Cells from 6 lines were completely ineffective in sensitizing their hosts to the C3H test grafts, and (2) those from 5 lines sensitized their hosts as effectively as similar doses of cells of C3H origin. This finding may be taken as proof that these 5 lines contain cells of murine origin. As stated (*see* first paragraph under "Test for Presence of Murine Transplantation Antigens") at the beginning this test is not an exclusive one—the negative results do not necessarily rule out the possibility that the cells concerned were derived from mice. They might have been derived from strains sharing no strong transplantation antigens with the C3H test donor strain.

Chromosome Studies

Suspensions of cells obtained by trypsinization or by scraping with a rubber policeman were seeded into a Petri dish containing glass slides with 18 ml. of medium (20% filtered calf serum and medium 199). These were incubated in a 5 percent CO_2 atmosphere at 37° C. for 2 to 4 days when colchicine was added to each dish (approximately 5×10^{-6} M). Spreading of the arrested metaphase cells was effected 8 to 10 hours later

by replacing the medium with either hypotonic Gey's BSS (*2*) or with 0.5 percent sodium citrate solution. After 30 minutes of such hypotonic treatment the cells were fixed with 1:3 acetic alcohol. The fixed material on glass slides was air-dried thoroughly (*20*), stained with 2 percent acetic orcein at 55° C., and then dehydrated for permanent mounting.

For the chromosome counts, metaphase spreads were represented by a circle, and subgroups, as in sectors of a pie, were counted repeatedly until confirmed. Those spreads which presented no ambiguity in interpretation were regarded as "exact" counts. Where alternate interpretations seemed possible, the count was scored as "plus or minus 1" or "plus or minus 2" and those of lesser quality were rejected. Only with the ARF line was there an insufficient number of good counts; half of these counts were of "plus or minus 3" quality. As all counts and karyological analyses were made from selected metaphase spreads, those which were multiples of the stem type were under represented. These secondary modes did not constitute more than a small percentage of the total.

In those spreads suitable for chromosome morphology the number of telocentric (T) and of nontelocentric (non-T) chromosomes were counted. The criteria for chromosome classes were, in general, those of Ising and Levan (*21*), wherein "an M chromosome has a median or sub-median centromere and an index of long arm to short of 1–1.9; S chromosomes have sub-terminal centromeres with arm index of 2–4.9; and T has a *nearly* terminal centromere, arm index 5 or more." The kinetochore of mouse chromosomes is perhaps more easily recognized as such; however terminal "knobs" or acrocentric arms can cause difficulty in any classification system (*22*), especially in human material. By our criteria the acrocentric chromosome pairs numbered 18, 19, 20, 21, and 22 of the human karyographs of Tjio and Puck (*23*) would be classed as telocentric. All M and S chromosomes were counted together as "nontelocentric" and borderline chromosomes where classed as "indeterminates." This class (I) represented a measure of interpretative error and was added to each of the two main classes (T and non-T) to derive the most conservative limit for each main class. The number of chromosomes and the metaphase analyses for each cell line and incidental observations are reported here.

HeLa.—Originally Hsu (*24*) observed a range in chromosome number from 70 to 130, with a mode of 80 to 90 chromosomes. Chu and Giles (*25*) in their analyses of clonal derivatives of HeLa cells reported 31 M, 43 S, and 4 T chromosomes in strain S1, and 30 M, 46 S, and 2 T in strain S3. In our analyses of 55 metaphases a modal range of 75 to 78 was seen; from 34 cells studied in detail a pattern of 53 to 63 nontelocentrics and 13 to 21 telocentrics was seen. Differences between these studies reflect the stricter definition of T chromosomes in the former study. The average stem complement of HeLa was 58 non-T, 16 T, 1½ I. In 6 cells either 1 or 2 "minutes" (fig. 1) were noted.

D-227.—In the present examination a mode of 62 to 64 chromosomes defined the complement, and the incidence of telocentrics was 8 to 14 per cell. Counts were made from 51 metaphase spreads, and the analysis of

chromosome types was made from 22 metaphases. A single "minute" chromosome was frequently noted. The characteristic formula derived for D–227 was 51 non-T, 11 T, 3 I (fig. 2).

McCoy-A.—In the present material, the modal range for 35 metaphase spreads was 70 to 74 chromosomes with 4 to 8 telocentrics (fig. 3) as determined from the analysis of 11 metaphase spreads. The average formula characterizing the stem-cell complement of the McCoy-A line is 53 non-T, 10 T, 8 I.

Amnion Ep.L.—Chromosome counts from 38 cells ranged from 70 to 80, with the mode at 75 to 78, which is similar to counts by Fernandes (*8*). Considerable variation was seen in the number of telocentrics. Two extreme cases had these formulas: 67 non-T, 0 T, 4 I and 71 non-T, 0 T, 4 I. In the remainder, the telocentrics varied between 2 and 18 per cell with an average distribution of 65 non-T, 7 T, 4 I (fig 4).

ARF.—A low proliferative capacity in this line and a poor response to the pretreatment made it necessary to consider less favorable spreads. This included 13 counts of ± 3 quality and 15 counts of ± 2 quality. Chromosome counts between 55 and 67 were obtained; nearly half of these occurred at 62 per cell. Among 17 cells analyzed further the least number of telocentrics occurred in a cell with 38 nontelocentrics, 12 telocentrics, and 5 indeterminates. The other extreme is represented by the formula 32 non-T, 29 T, 5 I. A cell of high ploidy (217 ± 4) contained 126 non-T, 85 T, 6 I; this proportion of nontelocentrics did not exceed the average, which was 35 non-T, 22 T, 6 I (fig. 5).

L strain (*NCTC clone 929*).—From 41 metaphase spreads the L cell showed chromosome numbers ranging from 62 to 70, with the mode at 64 to 66. Between 16 and 22 nontelocentrics per cell were noted in an analysis of 16 metaphase spreads; the characteristic formula for this line was 19 non-T, 45 T, 2 I. An occasional "minute" was noted. A long ruffled chromosome was present (fig. 6) similar to one described by Rothfels *et al.* (*1*) in their MCN strain. If identical, its presence would confirm the contention of these authors that their MCN strain was actually an L-cell contaminant (*1*).

MCN.—The chromosome number of this line was between 62 and 65 as determined from 30 counts. A range of 18 to 24 nontelocentrics per cell was found on examination of 14 selected cells. The characteristic formula for MCN is 21 non-T, 40 T, 2½ I (fig. 9).

Wistar 8.—In 44 counts of the Wistar 8 line the mode was 67 to 69 chromosomes per cell. Between 14 and 22 nontelocentrics per cell were observed; the average formula for Wistar 8 is 18 non-T, 47 T, 1½ I (fig. 8). These findings resemble the preceding analysis of the MCN line.

McCoy-B.—The mode for McCoy-B was 63 to 68 chromosomes for 28 cells counted, with 13 to 18 nontelocentrics per cell (fig. 7). An average formula of 15 non-T, 48 T, 3 I was derived from 19 selected metaphases. A long metacentric with a strong secondary constriction was clearly recognized in 6 of the 28 metaphases studied. The two constrictions, secondary and primary, mark the chromosome parts into the proportions

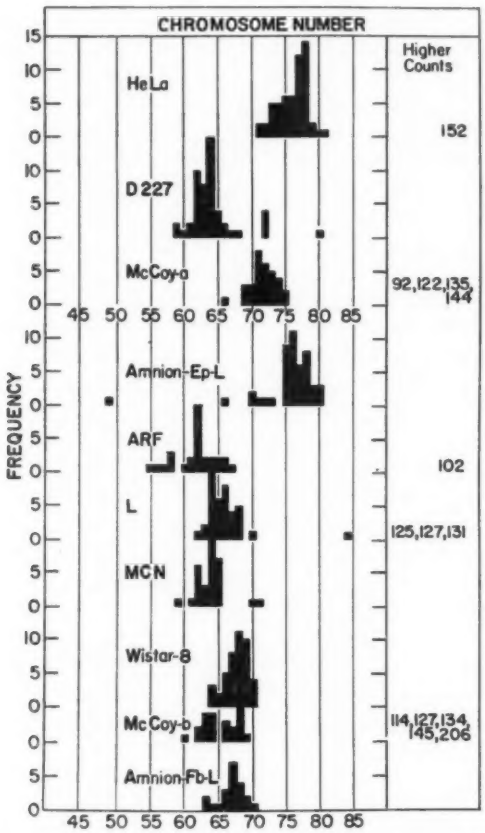
2:4:3, respectively (fig. 12). The position of these constrictions and their total length correspond well to the dicentric described in an L cell line, L-P55-N2(*2*). It is suggested that the same marker is also represented in Amnion Fb.L (fig. 11) and in a derivative of AMK clone 2-2 (fig. 10). This AMK line has retained the marker through a derivation involving a selection for a tenfold increase in tolerance to the antibiotic puromycin (*26*). These dicentric or diconstricted chromosomes, if identical, would indicate a common origin for McCoy-B, Amnion Fb.L, L cell (L-P55–N2), and AMK 2-2.

Amnion Fb.L.—Chromosome counts of 21 metaphases indicate a mode at 66 to 68. The implication from the presence of the 2:4:3 marker that the Amnion Fb.L is also a case of L cell contamination seems confirmed by its mode and a consideration of the centromeric types among its chromosomes. In 12 cells so analyzed, the average constitution was 19 non-T, 47 T, 1 I. The 2:4:3 chromosome (fig. 11) was recognized in approximately half of all cells studied.

Chromosome counts are compared in the histograms of text-figure 1. All 11 cell lines, except Det-504, may be termed heteroploids or subtetraploids. The term "heteroploid" is used collectively since all 4 of the species of stated origin for these lines have diploid numbers between 40 and 46 chromosomes. The Det-504 line is a relatively unaltered line since it is diploid or nearly so according to counts by Stulberg, Berman, and Shapiro (*5*), who found 27 diploid cells at 46, 29 cells at 2n-1, and a few counts at 44 and 47. From all the chromosome counts presented in text-figure 1 we can point to only two general groupings: HeLa, McCoy-A, and Amnion Ep.L have modes between 70 and 80; the others, D-227 ARF, L, MCN, Wistar 8, McCoy-B, and Amnion Fb.L, show slight differences, but their modes lie between 60 and 70. The more pertinent results concern the types of chromosome with respect to centromeric position. Of the lines examined (table 4), HeLa, D-227, McCoy-A, and Amnion Ep.L showed an obvious predominance of nontelocentric types. One line, the ARF cell, displayed a more intermediate frequency of nontelocentric chromosomes, the limits being 56 to 66 percent. The remaining lines, L, MCN, Wistar 8, McCoy-B, and Amnion Fb.L, constitute a group with a low frequency of nontelocentric types. These three groupings do not overlap to any extent (table 4).

DISCUSSION

In previous work (*13*, *14*) the spectrums of activity of cytotoxic antibodies produced with antigens rich in nucleoprotein fraction from HeLa, H.Ep#1, and MCN cell lines were not species specific, but appeared related to the morphology of the cell tested. Thus HeLa and H.Ep#1 antiserums were cytotoxic for epithelial-like cells reported to be of human or rabbit origin, and MCN antiserum was cytotoxic for fibroblast-like cells allegedly of rat, mouse, rabbit, human, or bovine origin. In the



TEXT-FIGURE 1.—Histograms for chromosome numbers of 10 cell lines.

TABLE 4.—Summary of karyological characteristics of cell lines; frequencies of types of chromosomes

Cell strain	Modal chromosome number*	Average chromosome complement			Proportion of nontelocentrics† (*percent*)
		Nontelocentric	Telocentric	Indeterminate	
HeLa	75–78	58	16	1½	77–79
D-227	62–64	51	11	3	79–84
Det-504‡	45–46	—	—	—	—
McCoy-A	70–74	53	10	8	74–85
Amnion Ep.L	75–78	65	7	4	85–90
ARF	60–64	35	22	6	56–66
L	64–68	19	45	2	29–33
MCN	62–65	21	40	2½	33–37
Wistar 8	67–69	20	47	1	30–32
McCoy-B	63–68	15	49	2½	23–27
Amnion Fb.L	66–68	19	47	1	28–30

*Modal range of chromosome number is defined as the smallest interval that includes at least two thirds of the counts.

†Lower limit (%) from distinctly recognizable nontelocentrics only; higher limit computed by lumping all indeterminates with nontelocentrics.

‡Det-504 counts by courtesy of Stulberg, Berman, and Shapiro (*5*).

present study different results have been obtained with other cell lines. Three cell lines of human origin, D-227, Det-504, and McCoy-A, although of fibroblast morphology, were susceptible to the HeLa antiserum. The spectrum of the MCN antiserum was not modified. From the literature it appears that, in general, antiserums produced against tissue-culture cell lines are species specific (*27–30*), though the range of the combinations tested is small. However Habel *et al.* (*31*) have found that the inhibitory effect of cellular antiserums on virus cytopathogenicity is not species specific in the sense that antiserum against a human cell line was effective against monkey kidney cells *in vitro*. Similarly, Quersin-Thiery (*32*) has reported that monkey-kidney culture antiserum can agglutinate several human cell lines but not others of supposedly human origin; antiserums against L cells cross-reacted weakly with HeLa cells. Cross-reactions based on complement fixation between antiserums of cell lines of several species have been reported also by Coriell *et al.* (*33*).

The significance of these results is questionable at the present time as the actual identity of several of the cell lines tested has been challenged. It is therefore difficult to decide whether agglutinating and cytotoxic antibodies produced against cell lines are species specific. Definition of the numerous antigens involved in the formation of these antibodies should make this analysis more clear. Cells grown *in vitro* apparently lose organ-specific antigens within 3 to 4 days (*34*). Primary cultures of human fetal cells have been found to retain blood-group antigens at least from 28 to 35 days (*35*). In our experience (table 1) HeLa nucleoprotein antiserums agglutinated human red cells of group O-A-B at approximately the same titer, confirming the results obtained by Habel *et al.* (*36*) with whole-cell antiserums.

The present finding that HeLa nucleoprotein antiserum agglutinates human red cells and that MCN antiserum hemolyzes murine red cells conforms with the claim that hemagglutinating antibodies characterize the species of a cell strain (*37*). It also appears that hemagglutinating and hemolyzing antibodies differ from the antibodies responsible for the cytotoxic effect.

The analysis of transplantation antigens present in a cell line can provide decisive evidence of its origin as indicated by the data presented here. At least 15 different loci are involved in determining the transplantation antigens of the mouse, and at some of these, multiple alleles have been observed (*38, 39*). These antigens are not all of equal strength, and a disparity between donor and recipient with respect to a single strong antigen is sufficient to procure the breakdown of a skin graft within about 2 weeks. Transplantation antigens in common have not been observed in members of different species. In our experiments primary cells from 4 species, monkey, mouse, rat, and rabbit, certainly had no detectable transplantation antigens in common with C3H mice. Hence the accelerated rejection of a skin homograft transplanted to a mouse previously inoculated with living tissue cells from an unknown source may be taken as valid evidence that the inoculated cells and the test graft

must have possessed at least one murine transplantation antigen, probably an important one, *in common*. Such evidence does not, however, afford proof that the inoculated cells and the skin test graft have identical immunogenetic constitutions because of the multiplicity of transplantation antigens. The findings indicate (table 3) that at least some portion of the cell population in five of the lines tested must have been of murine origin. Moreover the intensity of the host reaction against the test grafts in these cases suggests that if the cell cultures concerned comprised a mixed population of murine and nonmurine cells, upward of a million murine cells must have been present in the inoculums (*40*). None of these cell lines represented a mixture of cells of different types, by morphological and serological criteria, as in a mixture of cell lines previously described (*7*). When the cells failed to sensitize their host, probably they were not of murine origin. On the basis of these results it appears that the induction of sensitization to test skin homografts can provide a clear indication of the origin of a cell line. The application of this method is unfortunately limited to species in which inbred strains are available and to certain combinations of strains in which the transplantation antigens are of appropriate strength. Other limitations of the method have already been discussed.

The significance of the susceptibility to poliomyelitis virus as a cell marker originates from the general observation that only epithelial-like cells of human origin in established cell lines have been found to support the growth of this virus. By contrast, established fibroblast-like cells of any species are resistant to poliomyelitis virus. Although exceptions to this generalization have been reported for a monkey-heart line (*41*), some rabbit lines (*42*), a chicken-heart line (*43*), and one guinea-pig line (*44*), the actual origin of these cells has lately been questioned. Our findings do not contradict the general rule. The only exception we have found is the growth of poliomyelitis Type I in Det-504 cells of human origin and of fibroblast morphology. However Det-504 cannot be classified with certainty as an established cell line since unfrozen samples survived for only 20 to 30 passages, and the cells have retained the morphological characteristics of a primary fibroblast. In any case, susceptibility of a cell to a certain virus cannot constitute a "marker" of the species of origin until the examination of several clearly defined cell lines bears out its value.

The identification of cells from their chromosome morphology and constitution is difficult unless conveniently recognizable chromosomes exist. Except for unusually distinct markers, certainty regarding correspondence in two different cell materials is debatable. Furthermore in tissue-culture cell lines there is a notable pleomorphism in their karyological complement during establishment (*45*), and shifts in stem cell occur under various unknown selection pressures (*46*). However where differential diagnosis is desired between species whose original diploid nature is markedly different, a distinct advantage is available. This is most obvious in mouse and human cells. In the mouse there are 40 telocentrics

and no nontelocentrics (M + S), whereas by our broad definition, there are no more than 10 telocentrics in the total 46 in man (*23*). The same consideration applies to a rabbit-mouse differential comparison, as the rabbit has 8 telocentrics and 36 nontelocentrics (2n = 44), according to J. Clausen's study (*47*).

The creation and accumulation of nontypical chromosomes as metacentrics in murine material does occur in tissue culture (*48*); and conversely, new telocentrics are seen in human material (*49*). However there seems to be a tendency to conserve the general types of chromosomes, as noted by Levan (*49*) in certain human tumors.

The evidence presented from chromosome cytology appears consistent with the view that the first 5 lines of supposedly human origin (table 4) are correctly identified. The retention of the 2n condition in the Det-504 line and the predominance of the nontelocentric types among HeLa, D-227, McCoy-A, and Amnion Ep.L support this interpretation.

Likewise, the stated rat origin of the ARF line seems verified by the intermediate proportion (56–66%) of nontelocentrics found. The normal rat has 43, 52, or 62 percent of its diploid complement in the nontelocentric class, depending on which of three interpretations one considers. For the rat, Makino and Hsu (*50*) described 18 chromosomes as metacentric and 24 as telocentric; Tjio and Levan (*51*) described 22 and 20 chromosomes in these respective classes; and Ohno and Kinosita (*52*) found 26 and 16. Chromosome classes in the first work cited (*50*) correspond closely to our criteria.

The remaining 5 cell lines, L, MCN, Wistar 8, McCoy-B, and Amnion Fb.L are indistinguishable, as all have approximately one third of their chromosomes in the nontelocentric class and have similar modes. Only the L line is said to have a mouse origin and the occurrence of about 19 metacentrics is similar to other findings (*2*). This suggests that each of these 4 lines represents an independent contamination by the L cell, and the presence of the 2:4:3 marker tends to support this. On the contrary, to accept the stated species of origin—human for MCN, McCoy-B, and Amnion Fb.L, and rabbit for Wistar 8—seems to require acceptance of an extensive and rather directed chromosomal restructuring that stabilizes surprisingly at the L prototype.

From all these considerations it appears that the immunological and karyological criteria can give sufficient information concerning the origin of a cell line. No criterion alone is sufficient except in special cases, but together, the criteria may corroborate each other. Thus transplantation antigens can only be identified in the mouse and, possibly, in the rat, guinea pig, and Syrian hamster, since inbred strains of these species are available (*53*). Cellular antiserums, although indicative, have not yet been produced against any definite antigen of the mosaic seen on the cell surfaces or on internal structures; karyological data are helpful when the original diploid complements are characteristically distinct so as to permit recognition within certain probability limits after chromosomal restructuring. Morphological type and virus sensitivity, although historically

TABLE 5.—Summary of test results

Cell line	Stated origin	Morphology	Antiserum susceptibility	Predominant type of chromosome	Sensitivity to poliovirus	Presence of mouse antigen
HeLa	Human	Epithelial	HeLa	Nontelocentric	+	−
D-227	Human	Fibroblast	HeLa	Nontelocentric	−	−
Det-504	Human	Fibroblast	HeLa	Nontelocentric	+	−
McCoy-A	Human	Fibroblast	HeLa	Nontelocentric	−	−
Amnion Ep.L	Human	Epithelial	HeLa	Nontelocentric	+	−
ARF	Rat	Fibroblast	Neither	Near equal	−	−
L	Mouse	Fibroblast	MCN	Telocentric	−	+
MCN	Human	Fibroblast	MCN	Telocentric	−	+
Wistar 8	Rabbit	Fibroblast	MCN	Telocentric	−	+
McCoy-B	Human	Fibroblast	MCN	Telocentric	−	+
Amnion Fb.L	Human	Fibroblast	MCN	Telocentric	−	+

the first criteria to be considered, have not been sufficiently analyzed to give more than a general impression of the cell-line characteristics.

Table 5 brings together the results of all tests performed on the 11 cell lines. It appears that for HeLa, D-227, Det-504, McCoy-A, Amnion Ep.L, ARF, and L there is no contradiction between the stated origin and the karyological and immunological results. The other cell lines—MCN, Wistar 8, McCoy-B, and Amnion Fb.L—are indistinguishable from the L line by all the criteria considered. Alternative hypotheses are that these 4 cell lines represent contaminations from L cells and are actually L, as has been suggested in other reports (*1*, *2*), or that these cell lines have converged, independent of their origin, on a common cellular pattern similar to the L cell.

The latter hypothesis implies that 3 lines of human and 1 line of rabbit origin must each have acquired or unmasked specific transplantation antigens in common with the mouse during the process of adaptation to growth *in vitro*, and that each must have converged on a common chromosome pattern. If this were true it would be most remarkable. The former hypothesis would then seem more acceptable, involving only the recognition that L cells have exceptional ability to survive under less than optimal conditions and that human error may have occurred in each instance.

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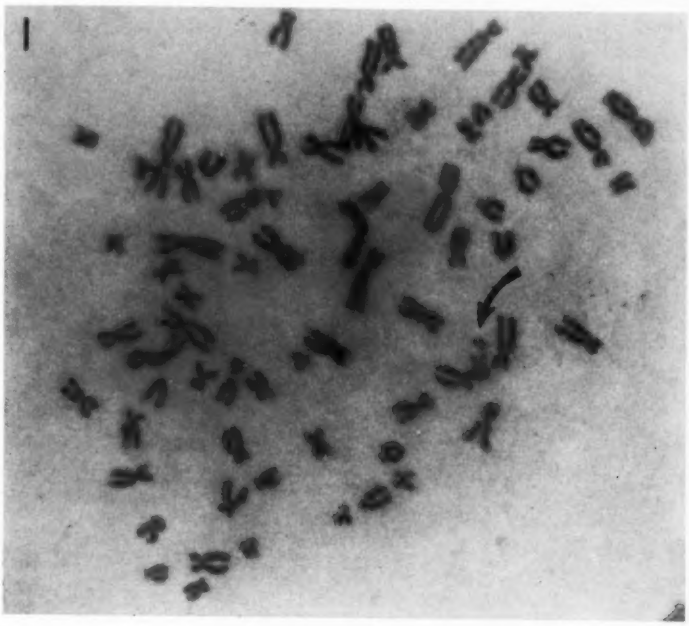
Photomicrographs of metaphase spreads to show representative chromosome number and form. Light microscopy, air-dried (except fig. 10), acetic-orcein stained.

PLATE 36

FIGURE 1.—Metaphase from HeLa cell with 74 chromosomes. *Note* "minute" (57 non-T, 16 T, 1 m.). × 1,200

FIGURE 2.—Metaphase from D-227 cell with 62 chromosomes. *Note* "minute" (53 non-T, 8 T, 1 m.). × 1,400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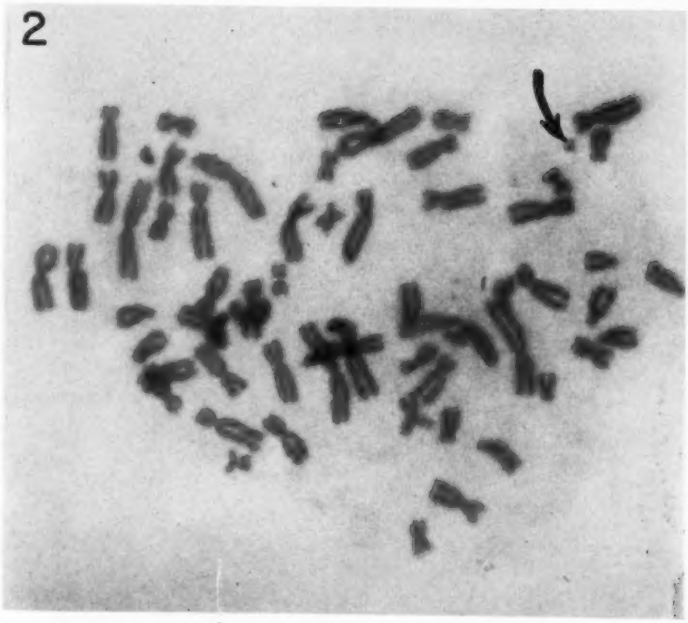
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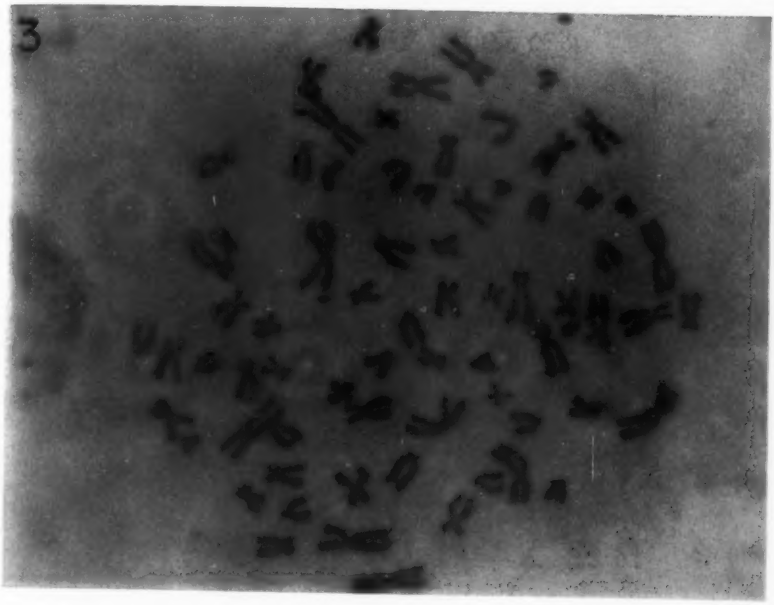
PLATE 37

FIGURE 3.—Metaphase from McCoy-A cell with 72 chromosomes (55 non-T, 17 T). × 1,650

FIGURE 4.—Metaphase from Amnion Ep.L cell with $76^{\pm 2}$ chromosomes (62 non-T, 11 T, 3 I). × 2,100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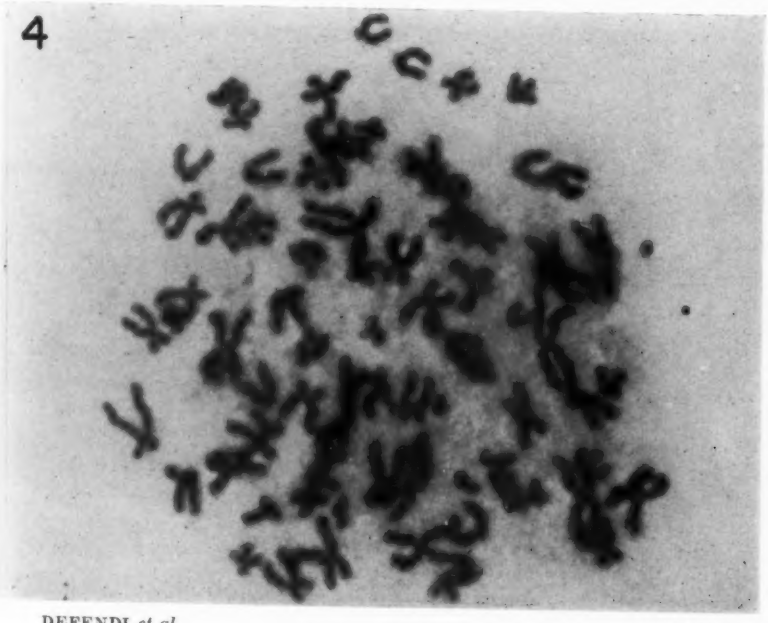
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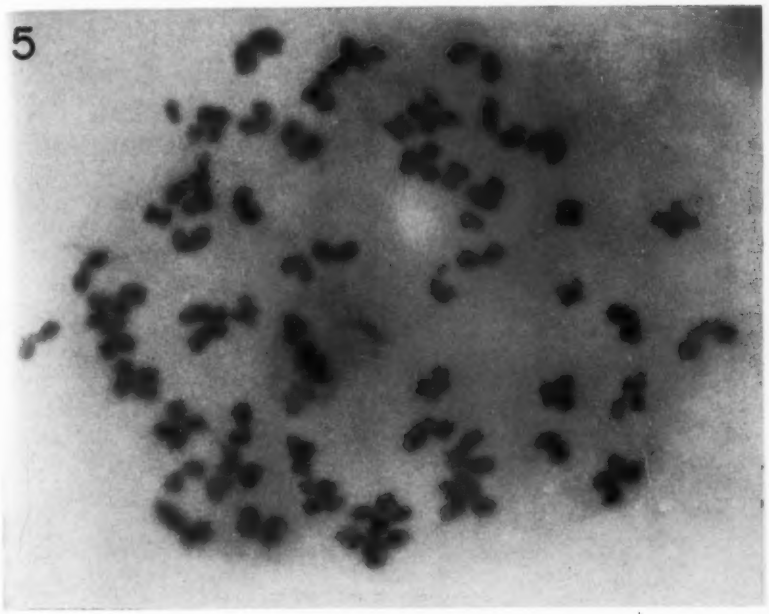
PLATE 38

FIGURE 5.—Metaphase from ARF cell with $64^{\pm 2}$ chromosomes (37 non-T, 20 T, 7 I). × 2,300

FIGURE 6.—Metaphase from L cell with 65 chromosomes (2 chromosomes are not in the field of this fig.). *Note* long ruffled chromosome and one "minute" (18 non-T, 46 T, 1 m.). × 1,650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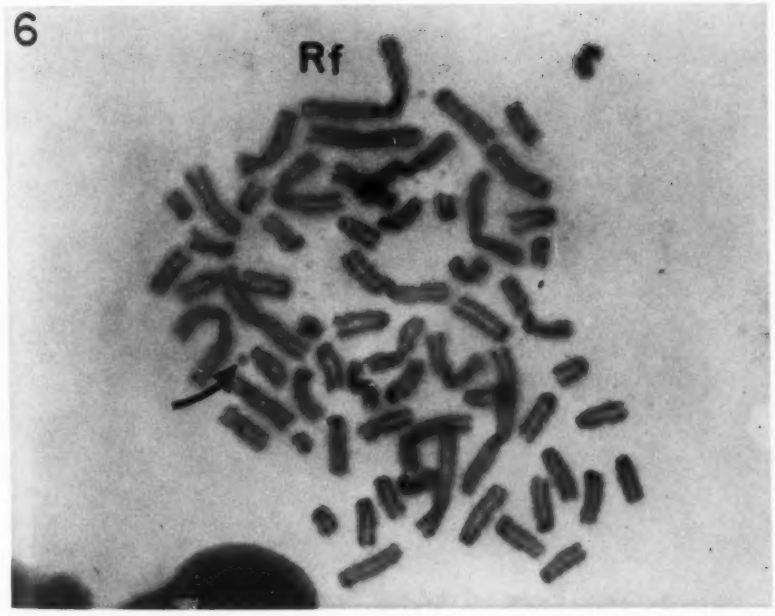
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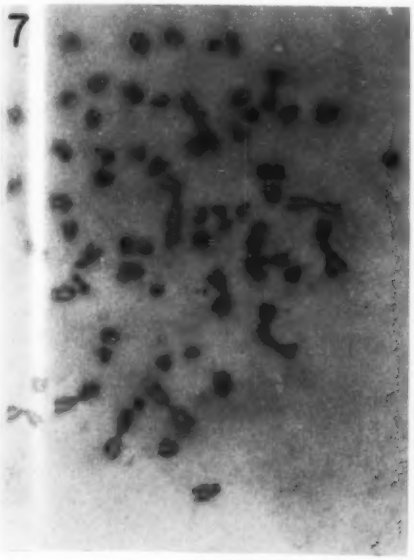
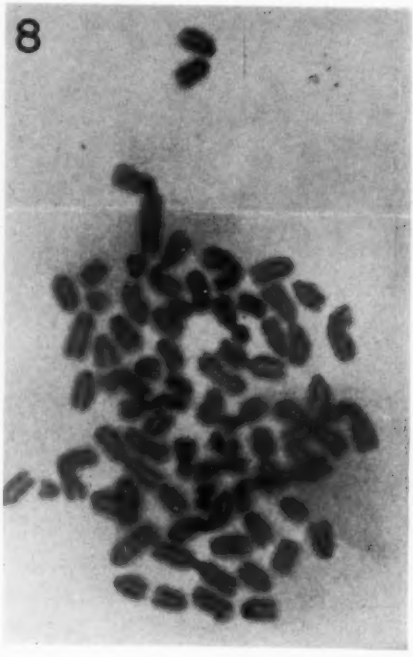
PLATE 39

FIGURE 7.—Metaphase from McCoy-B cell with 64 chromosomes (17 non-T, 47 T). × 1,000

FIGURE 8.—Metaphase from Wistar 8 cell with 66 chromosomes (22 non-T, 44 T). × 1,100

FIGURE 9.—Metaphase from MCN cell with 64 chromosomes (21 non-T, 42 T, 1 I). × 1,650





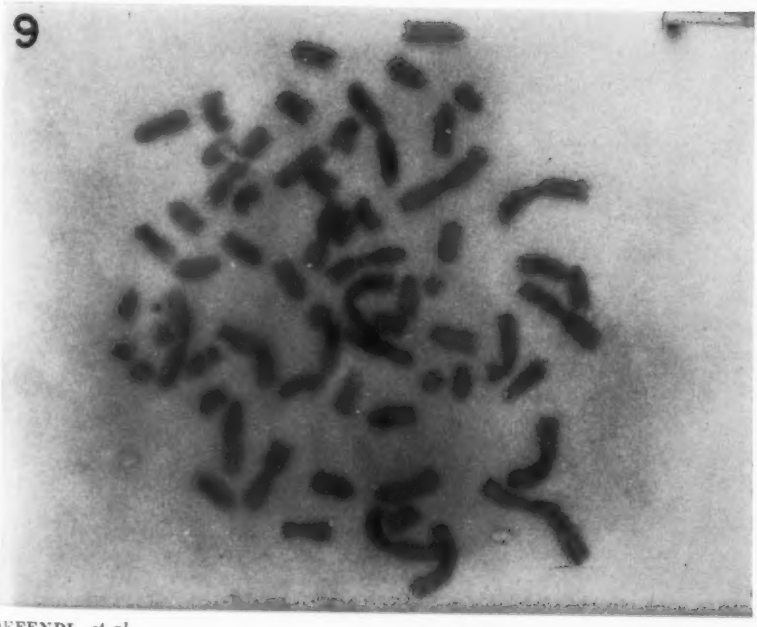


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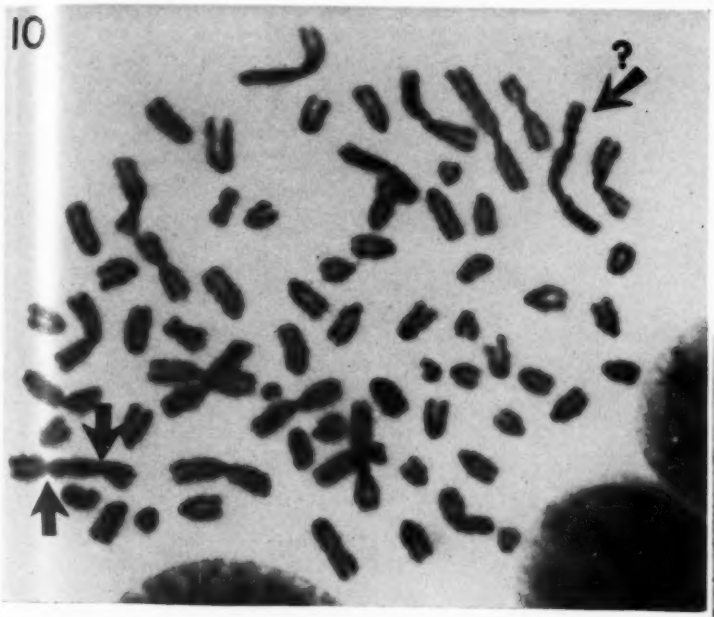
PLATE 40

FIGURE 10.—Squash metaphase from cell of AMK 2-2 ("10×-Puro" derivative) line with one 2:4:3 dicentric and probable mate (?). Cell has 69 chromosomes (21 non-T, 48 T). × 1,900

FIGURE 11.—Metaphase from Amnion Fb.L cell showing one 2:4:3 arm ratio dicentric. Cell has 65 chromosomes (20 non-T, 45 T). × 1,350

FIGURE 12.—McCoy-B cell with pair of chromosomes with secondary constriction giving proportions of 2:4:3. × 2,000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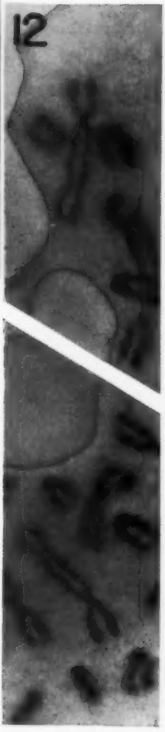
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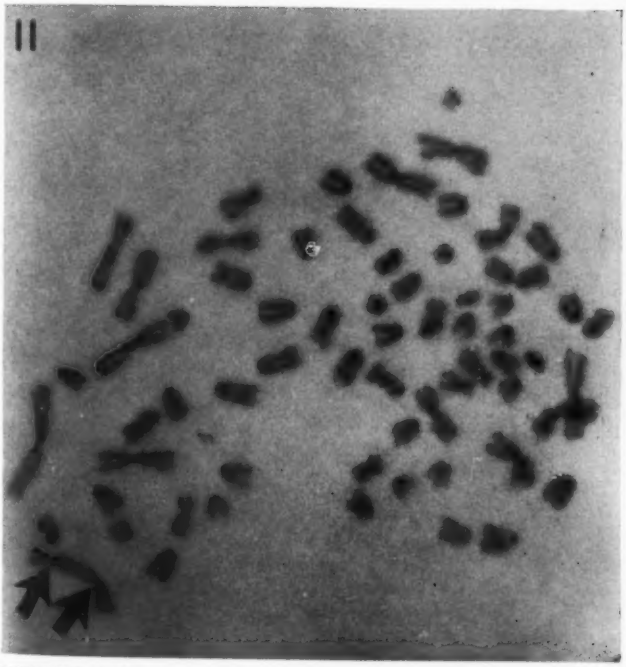
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Relative Carcinogenic Activity of Monomethyl Derivatives of Benz[a]anthracene in Fischer Line 344 Rats [1,2]

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SUMMARY

All 12 monomethyl derivatives of benz[a]anthracene were tested for carcinogenic potency by bilateral subcutaneous injection of 2 mg. in 0.2 cc. of paraffin in each of 60 Fischer line 344 rats. Four of the compounds (10-, 4-, 5-, 9-methyl benz[a]anthracene) were highly carcinogenic. Five of the derivatives (7-, 6-, 4′-, 3-, and 1′-methyl benz[a]anthracene) were weakly active and the remaining 3 (3′-, 2′-, and 8-methyl benz[a]anthracene) were inactive. The induced neoplasms were observed with almost equal frequency and in approximately the same average latent period in males and females. Ninety percent of the 248 induced neoplasms were classified as fibrosarcomas or sarcomas of myogenic origin. These were the most frequent types induced by each of the active compounds.—J. Nat. Cancer Inst. 25: 387–391, 1960.

THE SEARCH for the physical and chemical properties of hydrocarbons that are correlated with carcinogenic activity has been limited by the paucity of dependable observations on the potency of the hydrocarbons. Inherent difficulties in the attempt to define the degree of carcinogenic potency of the hydrocarbons have been summarized by Hartwell (*1*). These include variations in the species and genetic constitution of the host, differences in dose and method of application of the chemical, and lack of definition of the endpoint.

Recently all 12 of the monomethyl derivatives of benz[*a*]anthracene were synthesized and made available for testing. These have been tested by von Haam, as reported by Newman and Otsuka (*2*), by skin and parenteral administration in pure-bred C3H mice and in our laboratory by subcutaneous injection in inbred Fischer line 344 rats. There are some significant differences in response that may be related to the difference in species or the method of administration of the chemical. In each case the entire series of analogues were tested by the same method in animals of the same genetic constitution.

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[1] Received for publication March 1, 1960.

[2] Supported by research grant C-1864 from the National Cancer Institute, National Institutes of Health, Public Health Service.

553742—60——16

MATERIALS AND METHODS

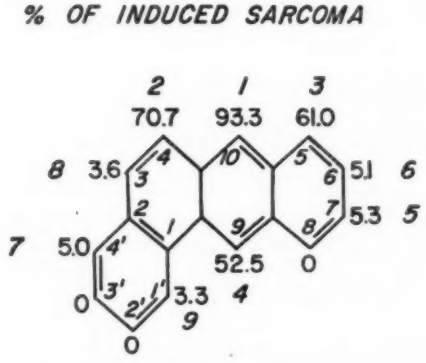
Approximately 250 mg. of each compound was supplied through the courtesy of Dr. Melvin S. Newman, of Ohio State University. Each sample was dissolved in 5 cc. of warm benzene to which was added 25 cc. of melted paraffin (melting point 48° C.) and maintained in a water bath at 75° C. until all odor of benzene was dissipated. Each of 60 rats was injected bilaterally in the subcutaneous tissues with 0.2 cc. of the warm paraffin solution containing 2 mg. of the hydrocarbon at each injection site. The dose for each rat was 4 mg.

The rats were all pedigreed Fischer line 344 rats between 3 and 4 months of age at the time of injection. Each chemical was injected in approximately 30 males and 30 females.

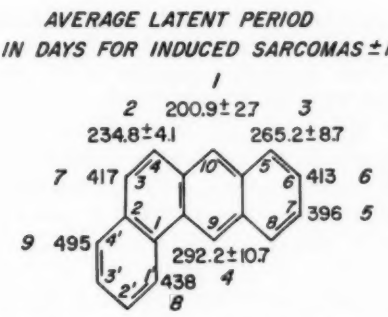
After the injection the paraffin solution solidified in fairly uniform ellipsoids that were easily palpable. The rats were examined weekly and the time of observation of the first perceptible increase in size was recorded. At necropsy the gross tumors were described and the nodules were removed and preserved for microscopic examination. Each tumor and nodule was examined to establish the presence of neoplasia and classify the malignant disease.

RESULTS

The results are summarized in tables 1, 2, and 3. Most of the data presented in table 1 were previously reported (*3*). These data show that 4 of the compounds (10-, 4-, 5-, and 9-methyl benz[*a*]anthracene) were highly carcinogenic. Five compounds (7-, 6-, 4′-, 3-, and 1′-methyl benz[*a*]anthracene) were weakly active and 3 (3′-, 2′-, and 8-methyl benz[*a*]anthracene) were inactive. From 52 to 93 percent of the rats in the first group developed 1 or 2 neoplasms, while tumors were observed in only 3 to 5 percent of the rats in the second group. The neoplasms in



TEXT-FIGURE 1.—Relative position of the percentage of sarcomas induced by each of the compounds.



TEXT-FIGURE 2.—Relative position of the average latent period observed for the sarcomas induced by each of the compounds.

the rats of the first group occurred in a significantly shorter average latent period (201 ± 2.7 to 292 ± 10.7 days) than those of the second group (396 to 495 days). These data are shown graphically in text-figures 1 and 2.

TABLE 1.—Comparative carcinogenic activity of benz[a]anthracenes

Compound	Number of rats	Percent with tumors	Number of tumors	Mean latent period ± probable error in days
10-Methyl	60	93	97	201 ± 2.7
4-Methyl	58	71	58	235 ± 4.1
5-Methyl	59	61	41	265 ± 8.7
9-Methyl	61	52	39	292 ± 10.7
7-Methyl	57	5	3	396
6-Methyl	59	5	3	413
4'-Methyl	60	5	3	495
3-Methyl	55	4	2	417
1'-Methyl	60	3	2	438
3'-Methyl	54	0	0	
2'-Methyl	61	0	0	
8-Methyl	58	0	0	
Total	702	25	248	

One compound, 10-methyl benz[a]anthracene appeared to be more active than the other 3 highly active compounds. This is shown by a significantly higher percentage of rats with tumor and a significantly shorter average latent period. This compound, as previously reported (*3*), was more active than dibenz[a,h]anthracene and less active than methylcholanthrene or benzo[a]pyrene when tested at the same dosage level in rats by the same method. The other 3 active compounds were not significantly different from each other.

Table 2 shows that the tumors occurred with nearly equal frequency and in approximately the same average latent period in rats of both sexes. For each of the highly active compounds there was a slightly higher percentage of males than females with tumors. The differences, however, were not certainly significant. The frequency of rats with single and

double tumors also appeared to be fairly equally distributed in males and females. The proportion of rats that developed tumors at both injection sites decreased with decreasing potency of the chemical. For the most active compound 10-methyl benz[*a*]anthracene, there were 15 rats with 1 tumor and 41 with 2 tumors. Of the former, 8 were males and 7 were females, and among the latter, 22 were males and 19 were females. For the next most active compound, 4-methyl benz[*a*]anthracene, there were 24 (14 males and 10 females) with 1 tumor and 17 (10 males and 7 females) with tumors at both sites of injection. Of the rats injected with 5-methyl benz[*a*]anthracene, 31 (17 males and 14 females) had single tumors and only 5 (2 males and 3 females) had 2 tumors. For 9-methyl benz[*a*]anthracene there were 25 rats with single tumors (14 males and 11 females) and 7 (4 males and 3 females) with 2 tumors.

TABLE 2.—Comparative response of males and females to the active benz[*a*]anthracene

	Males		Females		
Compound	Number of rats	Percent with tumors	Number of rats	Percent with tumors	Difference ± probable error
10-Methyl	30	100	30	87	13 ± 4.3
4-Methyl	29	83	29	59	24 ± 8.1
5-Methyl	28	68	31	55	13 ± 8.6
9-Methyl	32	56	29	48	8 ± 8.6
Weakly active	150	5	142	4	1 ± .75
Total	269	51	261	43	8 ± 2.9

	Males		Females		
Compound	Number of tumors	Average latent period in days mean ± probable error	Number of tumors	Average latent period in days mean ± probable error	Difference ± probable error
10-Methyl	52	190 ± 3.3	45	213 ± 3.9	−23 ± 4.4
4-Methyl	34	232 ± 6.0	24	239 ± 5.1	−7 ± 7.9
5-Methyl	21	253 ± 12	20	280 ± 12	−27 ± 17
9-Methyl	22	314 ± 16	17	264 ± 12	50 ± 20

Variation in the latent period increased with decreasing potency of the compound but did not appear to be different in males and females. For the most active compound the difference between 190 ± 3.3 days for tumors in males and 213 ± 3.9 days for tumors in females is statistically significant ($P = 0.07$). For the next most active compound, the tumor occurred in males in an average of 232 ± 6.0 days and in females in 239 ± 5.1 days. There was a difference of 27 ± 17 days between the mean latent period in males and females that received 5-methyl benz[*a*]anthracene and 50 ± 20 days in the opposite direction for tumors in males and females that received 9-methyl benz[*a*]anthracene.

Table 3 shows the classification of the induced tumors. Fibrosarcomas were the most frequent type induced by each of the active compounds. Tumors of myogenic or probable myogenic origin were the next most frequent type. They were observed in each group. Likewise, the

extraskeletal bone-forming neoplasms and mixed tumors appeared to be randomly distributed.

TABLE 3.—Classification of neoplasms induced by active benz[a]anthracenes

Classification	10-Methyl	4-Methyl	5-Methyl	9-Methyl	4 Weakly carcinogenic	Total
Fibrosarcoma	78	45	32	27	9	191
Rhabdomyosarcoma	2	4	1			7
Sarcoma—probably myogenic	9	5	2	9	1	26
Chondrosarcoma	1			1		2
Osteosarcoma	1	1	1	1		4
Mixed tumor (adenocarcinoma and sarcoma)	2					2
Mixed tumor (squamous-cell carcinoma and sarcoma)	2					2
Liposarcoma		1				1
Angiosarcoma			1			1
Sarcoma—not classified	2	2	4	1	3	12
Total	97	58	41	39	13	248

DISCUSSION

These results appear to agree with the results of von Haam, as reported by Newman (*2*), except for the relative position of the 9-methyl- and 6-methyl-compounds. In C3H mice 9-methyl benz[a]anthracene was only moderately carcinogenic and 6-methyl benz[a]anthracene was highly carcinogenic. The position of these 2 derivatives appears to be reversed in Fischer line 344 rats. Four other compounds (7-, 4′-, 3-, and 1′-methyl benz[a]anthracene) that were weakly active in Fischer line 344 rats appeared to be inactive in C3H mice. These may be species differences in response or differences resulting from the mode of administration of the chemical.

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Electron Microscopy of Milk Secretion in the Mammary Gland of the C3H/Crgl Mouse. I. Cytomorphology of the Prelactating and the Lactating Gland [1,2]

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SUMMARY

The cytology of milk secretion in the mammary gland of the mouse was examined by light and electron microscopy. Protein, fat, and glycoprotein, 3 of the major milk components, were identified by histochemical methods and shown to increase in quantity in the alveolar lumens through the successive stages of pregnancy and lactation. Protein and fat occurred as 2 different kinds of droplets which were readily identified in electron micrographs. The relative quantity of each kind of droplet observed at any given stage of pregnancy or lactation was positively correlated with the quantity observed by histochemical methods. The protein droplets appeared to be formed in the vacuolar spaces of the Golgi apparatus. The Golgi apparatus and the endoplasmic reticulum were markedly hypertrophied in lactating cells. The mode of origin of the fat droplets in cytologic terms remains unknown. The evidence indicates that protein and fat droplets are released into the alveolar lumen without the fragmentation of large masses of apical cytoplasm.—J. Nat. Cancer Inst. 25: 393–421, 1960.

THE MAMMARY gland of the C3H/Crgl mouse has been extensively investigated during continuing research on the genesis of mammary cancer in this strain of mouse. Harkness *et al.* (*1*) reported that normal prelactating mammary gland and hyperplastic alveolar nodules were not distinguishable cytochemically. Pitelka *et al.* (*2*) examined mammary tissues of the C3H/Crgl mouse with the electron microscope; they reported that viruslike particles were frequently observed in adenocarcinomas and hyperplastic alveolar nodules, but were rare in normal prelactating tissue. Nandi (*3*) has reported the results of a detailed study of endocrine control in the development and function of the mammary gland of the mouse. Recent studies by DeOme *et al.* (*4*) reported the probable precancerous nature of the hyperplastic alveolar nodules of the C3H/Crgl

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mouse. For the most part, the cytomorphologic aspects observed in these earlier studies have not been examined with the electron microscope. The present report gives a description of the cytology of milk secretion in the C3H/Crgl mouse as seen with the electron microscope.

MATERIALS AND METHODS

The mammary-gland tissues used for light and electron microscopy were obtained from 3- to 4-month-old female mice from the C3H/Crgl strain. All mice were anesthetized intraperitoneally by sodium pentobarbital (*5*), and the following types of mammary tissue were surgically removed: tissue from nonpregnant virgin females; prelactating tissue from females that had been pregnant for 7, 14, and 19 days; and lactating tissue from females that were 4 and 15 days post partum. Three animals were killed for each stage of the secretory study, and numerous tissue samples from each animal were prepared.

Tissues for light microscopy were fixed and stained with the following: Bouin's fixative before staining with Masson's trichrome stain or periodic acid-Schiff (PAS) (*6*); Carnoy's fixative followed by the mercuric bromphenol blue stain for protein (*7*); and frozen sections of material were fixed in 10 percent neutral formalin and stained for lipides with Sudan IV.

For electron microscopy [prelactating and lactating mammary-gland material is difficult to prepare] tissue samples measuring less than 1 mm. in their greatest dimension were removed from the anesthetized animals, and immersed for 2 hours in 1 percent OsO_4, buffered at *p*H 7.4 with acetate-veronal and with added sucrose, 0.045 gm. per ml. (*8*). The tissue blocks were then dehydrated in a graded series of ethyl alcohols and infiltrated with *n*-butyl methacrylate. Polymerization was carried out at a temperature of 45° C. with the catalyst 2,4-dichlorobenzoyl peroxide. Thin sections were cut with a Porter-Blum microtome set at 50 mμ and equipped with a plate-glass knife. The sections were then mounted on Formvar-coated specimen grids and examined in a RCA EMU-2B electron microscope. Some thin sections were floated on a saturated solution of uranyl acetate according to the method reported by Watson (*9*). Uranyl acetate treatment provided improved contrast, but tissues treated in this manner were otherwise identical to untreated tissues.

RESULTS

Light Microscopy

Prelactating and lactating mammary tissue stained with Masson's trichrome revealed histologic features that were in general agreement with previous descriptions by other workers (*10–13*). The mammary

tissue of 3- to 4-month-old virgin female mice consisted of scattered branching ducts embedded in adipose tissue (fig. 1). Low columnar or cuboidal ductal cells appeared as single or occasionally as double layers. The epithelial cells themselves had distinct plasma membranes and pale-staining cytoplasm. The nuclei were uniform in size and each had a delicate chromatin net and 1 or 2 small dense nucleoli. The ductal lumens frequently contained a small quantity of secretory material which gave a positive reaction with the PAS and bromphenol blue stains, indicating the presence, respectively, of 1,2-glycols of high molecular weight (*14*) and of protein.

Prelactating mammary tissue from mice pregnant for 7 days (fig. 2) showed many new duct buds and a few small alveoli. There was a noticeable increase of connective tissue surrounding the ducts, and increased numbers of mitoses were seen among the epithelial cells of the growing end buds. As in the mammary tissue of virgin females, small amounts of PAS and bromphenol blue positive material were identified in the ductal lumens.

Prelactating mammary tissue from mice pregnant for 14 days had numerous well-formed lobules composed of alveoli (fig. 3). The alveoli were lined with single layers of low columnar and cuboidal cells bordering small lumens. Mitotic figures were frequently seen. Ducts and occasional alveoli contained small amounts of material which gave a positive stain with PAS and bromphenol blue. Some clear vacuoles were seen in the apical cytoplasm and alveolar spaces. Frozen sections of comparable tissue stained with Sudan IV contained similarly placed sudanophilic droplets, suggesting that the clear vacuoles were empty spaces remaining after the action of fat solvents used in preparing the tissues.

The mammary tissue from mice pregnant for 19 days had many enlarged alveoli, each lined by a single layer of cuboidal or columnar cells (fig. 4). Many of the alveolar lumens contained secretion that gave a positive reaction with PAS and bromphenol blue. In addition, numerous fat droplets were present in the apices of the alveolar cells as well as in the secretion in the lumens. Mitoses were less frequently seen than in tissues from earlier stages of pregnancy.

Lactating mammary tissue from animals 4 days after delivery was made up of lobules of closely packed alveoli which replaced almost all of the adipose tissue (fig. 5). The alveoli were distended with secretion, giving a positive reaction with PAS and bromphenol blue. Numerous droplets of fat were likewise present in the lumens. The epithelial cells were cuboidal to flattened and the number of cells containing fat droplets was smaller than that of the prelactating tissue of the mice pregnant for 19 days.

The lactating mammary tissue from animals 15 days post partum (fig. 6) was similar to the tissue from mammary glands of females 4 days post partum, except that the alveoli were further distended with milk. The milk within the alveolar lumens again gave a positive stain with bromphenol blue and PAS (figs. 7 and 8).

Electron Microscopy

Electron micrographs of mammary tissue from C3H/Crgl virgin females revealed scattered ducts lined with 1 or 2 layers of cuboidal cells (fig. 9). Cell membranes were distinct, and variable numbers of microvilli were present at the luminal surfaces. The cytoplasm contained great numbers of small particles, 100 to 150 Å in diameter, which appeared similar to the ribonucleoprotein particles described by Palade (*15*). Very little organized endoplasmic reticulum was present. There were a few small mitochondria, which were ovoid or rod-shaped, and their densely granular matrices partially obscured the characteristic internal cristae (*16*). The Golgi apparatus was relatively small and inconspicuous, was usually located in the apical region of the cells, and consisted of 1 or more groups of flattened sacs and small vesicles. Nuclei were characterized by peripheral condensations of chromatin and large nucleoli. Ductal lumens were empty or contained small amounts of amorphous granular material.

Prelactating mammary-duct epithelium from animals at the 7th day of pregnancy, generally displayed similar characteristics. Sections through duct buds (fig. 10) showed small compressed lumens which contained small amounts of granular debris. A few microvilli were usually present at the luminal surface. As in ducts from virgin mice, the cytoplasm was again filled with small ribonucleoprotein particles, which tended to obscure other details. Mitochondria were larger and showed more distinct internal cristae. The Golgi apparatus was inconspicuous.

Prelactating mammary tissue taken from mice at the 14th day of pregnancy revealed numerous alveoli with small lumens (figs. 11 and 12). The alveoli were lined by single layers of cuboidal and columnar cells which tended to assume a pyramidal shape. The apices of the cells and the secretory lumens contained a few secretory droplets which resembled the casein particles described by other workers (*17–21*). Although casein is the principal protein in cow's milk, Morton (*19, 20*) has presented evidence indicating that a lipoprotein particle of similar appearance is also present. Therefore it seems best to refer to these particles simply as protein particles or protein droplets.

In addition to the protein droplets, the cytoplasm of some of the cells contained fat droplets, recognized by their high electron density and homogeneous appearance. They showed no constant topographical relationship to the Golgi apparatus or other cell organelles. The endoplasmic reticulum was more abundant than in previous stages of pregnancy, and consisted of scattered flattened sacs whose outer surfaces were covered with numerous ribonucleoprotein particles. As in mammary tissue from virgin females and from mice pregnant for 7 days, numerous ribonucleoprotein particles were scattered throughout the cytoplasmic matrix. Mitochondria were relatively numerous in all parts of the cells and exhibited the characteristic double-limiting membranes and internal cristae. Although

somewhat more conspicuous than in previous stages, the Golgi apparatus was still relatively small.

Prelactating mammary tissue from mice pregnant for 19 days (figs. 13 and 14) was composed of numerous alveoli lined with single layers of cuboidal and low columnar cells. The alveolar lumens and the apices of the epithelial cells contained many fat droplets and numerous spherical protein droplets varying in diameter from 40 to 300 mμ. The endoplasmic reticulum was comparable in amount to that seen in the animals pregnant for 14 days, and the structural details of the mitochondria were easily distinguished. The Golgi apparatus (fig. 14) was larger than previously observed, was located in the apex of the cells and on both sides of the nucleus, and consisted of parallel arrays of flattened sacs and tubules with associated microvesicles. Some particles with the density and shape of small protein droplets were associated with the Golgi membranes.

Lactating mammary tissues from animals 4 and 15 days post partum resembled each other, but differed sharply from prelactating tissues. The distended alveolar lumens contained numerous protein and fat droplets, and the cell membranes bordering on the lumens frequently had many microvilli (figs. 15, 17, and 19). The apices of the epithelial cells contained fewer fat droplets than those of the prelactating tissue of mice pregnant for 19 days, and, occasionally, fat droplets were observed apparently in the process of being extruded into the alveolar lumens (fig. 17). Fat droplets in the alveolar lumens frequently were surrounded by either a complete or incomplete membrane (fig. 15). Shreds of cytoplasm sometimes adhered to the surfaces of fat droplets. The endoplasmic reticulum was abundant and highly organized, consisting of arrays of flattened sacs oriented parallel to one another. The sacs were especially abundant in the basal two thirds of the cells, and their outer surfaces were covered with numerous ribonucleoprotein particles. The number of ribonucleoprotein particles lying freely in the cytoplasmic matrix was greatly reduced in comparison with that of the prelactating tissue. Mitochondria (figs. 15 and 16) were large, with conspicuous cristae and moderately dense internal matrices.

The Golgi apparatus (figs. 16, 18, and 20) was highly developed in comparison with that in the prelactating tissue, and was composed of parallel arrays of flattened sacs, large vacuoles, and numerous microvesicles, as reported for many other types of cells (*22–24*). The vacuoles of the Golgi apparatus frequently contained one or more spherical droplets which corresponded in size and structure to the protein droplets observed in the cell apices and the alveolar lumens. The smallest protein droplets tended to be associated topographically with the Golgi apparatus. Large vacuoles with smooth surfaces containing several protein droplets were frequently seen in the cell apices. Occasionally these vacuoles were open at the cell surface (fig. 21) and were interpreted as being in the process of releasing their content of protein droplets into the alveolar lumens. There was no evidence which would indicate that secretory product formation involves the fragmentation and breaking off of large masses

of apical cytoplasm, as is commonly suggested in some histology books (*25–27*).

Viruslike particles similar to those previously described in the C3H/Crgl mouse (*2*) were occasionally seen in the epithelial cells and in the alveolar lumens (figs. 12 and 18). These particles were of characteristic size and structure and were easily distinguished from the protein droplets. Unlike the protein droplets, the viruslike particles did not show any constant topographic relationship to the Golgi apparatus.

DISCUSSION

Conspicuous fine structural changes occur in the epithelium of the mammary gland of the intact 3- to 4-month-old C3H/Crgl female mouse during late pregnancy and lactation. Bargmann and Knoop (*28*) and Hollman (*29*) observed similar changes in the lactating mammary gland of the rat and mouse.

The endoplasmic reticulum was inconspicuous in mammary tissues from virgin females and females in early pregnancy. During late pregnancy, occasionally, organized sacs of the endoplasmic reticulum appeared, but during lactation they were abundant and highly organized. The outer surfaces of these sacs in lactating cells bore numerous ribonucleoprotein particles. Palade and Siekevitz (*30*) have presented convincing evidence indicating that these particles contain the major portion of the cytoplasmic ribonucleoprotein. The well-established association of cytoplasmic ribonucleic acid with active protein synthesis (*31, 32*) suggests that the abundance of ribonucleoprotein particles in the lactating epithelium may be related to the synthesis of large quantities of milk proteins. In addition highly organized and plentiful endoplasmic reticulum has been reported in other glandular cells such as those of the pancreas and liver (*33, 34*).

The mammary epithelial cells of virgin mice contained relatively few mitochondria, which were small and poorly organized. They increased in number and became more complex in structure during late pregnancy. The cells of lactating tissue contained greater numbers of mitochondria than the cells of prelactating or virgin tissue; furthermore, the characteristic internal cristae were more easily seen. These mitochondrial changes could be interpreted as reflecting an increased need for high energy compounds to synthesize milk components.

The size and abundance of the protein droplets indicate that they may be identical with the casein particles described by other workers (table 1). Morton's observations (*19, 20*) suggest that cow's milk contains at least 2 kinds of protein particles, 1 composed of casein and the other of lipoprotein. These 2 types of particles could not be distinguished by electron microscopy. In this report the term protein droplets is used and includes both types of particles. During pregnancy and lactation the number of protein droplets in the alveolar spaces observed by electron microscopy

paralleled the increase of material stained with bromphenol blue observed by light microscopy. Since casein is the major protein in milk (*35*), the present observations suggest that the protein droplets are the major milk component stainable with bromphenol blue.

TABLE 1.—Size ranges or average diameters of casein particles measured from electron micrographs of milk, milk extracts, or tissue sections of mammary gland

Author	Animal	Size range or average diameter ($m\mu$)
Nitschmann, 1949 (*17*)	Cow	80–120
Hostettler and Imhof, 1951 (*18*)	Cow	58–780
Morton, 1953 (*19*)	Cow	<30–250
Huth, 1957 (*21*)	Cow	~102
	Human	~30
Bargmann and Knoop, 1958 (*28*)	Rat	150–300
Wellings *et al.*, 1960 (*See* p. 397)	Mouse	40–300

The Golgi apparatus increased in size and complexity during pregnancy. In the lactating tissue this organelle was very conspicuous and was located at the sides of the nucleus and in the apices of the cells. The smallest recognizable protein droplets usually were seen in the vacuolar spaces of the Golgi apparatus. This observation suggests that the formation of the protein droplets in mouse milk resembles that of the zymogen granules in the pancreatic acinar cells of the mouse, the melanin granules in malignant melanoma cells in humans, and the zymogen granules in the pituitary acidophils of the rat (*36*, *37*).

A number of light microscopists have suggested a possible relationship between the Golgi apparatus and the formation of milk (*10*, *38*, *39*). These authors reported that the Golgi apparatus of the lactating mammary gland was greatly hypertrophied. In recent electron-microscope studies, Bargmann and Knoop (*28*) and Hollman (*29*) have shown that the protein droplets first appear in the Golgi apparatus of the alveolar epithelial cells of the lactating mammary gland of the rat and mouse. The present study essentially confirms the observations of these workers.

The possible role of the Golgi apparatus in the formation of secretory products has been discussed by a number of workers. Kirkman and Severinghaus (*40*) suggested that the Golgi apparatus may function as a condensation membrane to concentrate into drops or granules products synthesized in other locations, which diffuse through cytoplasm. Sjöstrand and Hanzon (*22*) and Haguenau and Bernhard (*23*) suggested that components of the Golgi apparatus may be converted into secretory products, and Dalton and Felix (*24*) proposed that the Golgi apparatus may remove water from maturing secretory substances. These views essentially agree with the observations of one of the present authors (*36*, *37*). The available evidence suggests that the Golgi apparatus concentrates smaller particles which may be synthesized and transported by other cytoplasmic organelles such as the endoplasmic reticulum.

Fat droplets were readily identified in histological sections by a tinctorial

reaction with Sudan IV and in the electron microscope by their homogeneity and their characteristic high-electron density. The sudanophil droplets observed by light microscopy were judged identical with the characteristic fat droplets observed by electron microscopy since both appeared with equal frequency and size at any given stage of pregnancy or lactation. The fat droplets showed no constant topographical relationship to any cell organelle, and their origin in cytomorphologic terms remains unknown.

The PAS-stainable component of milk should consist of 1,2-glycols (*14*) of high molecular weight, probably glycoproteins. This material could not be definitely identified with the electron microscope, and the possibility exists that the PAS-stainable component is in the protein droplets as a carbohydrate moiety bound to protein.

Electron micrographs of luminal cell membranes revealed numbers of microvilli. The evidence suggests that the protein droplets accumulate in the cell apex inside of membrane-enclosed vacuoles which subsequently open at the surface to release their contents. Fat droplets sometimes appeared to be extruded directly through the cell membrane, and, occasionally, droplets in the lumen were enclosed by membranes, which were interpreted as being derived from the plasma membranes of the secretory cells during the process of fat extrusion.

The secretory process in the mammary gland of the mouse appears not to involve the breaking off of large masses of apical cytoplasm into the alveolar lumen. In this regard, the results agree with the observations of Bargmann and Knoop (*28*) and Hollman (*29*).

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Figures 1 through 8 are light micrographs of histological sections of mouse mammary gland from virgin, pregnant, and lactating C3H/Crgl mice

PLATE 41

FIGURE 1.—Mammary gland of a 3- to 4-month-old virgin mouse showing scattered ducts and abundance of adipose tissue. Masson's stain. × 250

FIGURE 2.—Mammary gland of mouse on 7th day of pregnancy. Large and small ducts and several new duct buds (B). Masson's stain. × 250

FIGURE 3.—Mammary gland of mouse on 14th day of pregnancy; numerous small alveoli, some containing secretion. Masson's stain. × 250

FIGURE 4.—Mammary gland of mouse on 19th day of pregnancy. One half or more of fatty tissue has been replaced by new ducts and alveoli; some alveoli contain secretion. Many fat droplets (F) in cell apices and alveolar lumens; fat droplets appear as empty spaces due to action of lipide solvents in preparation of the tissue. Masson's stain. × 250

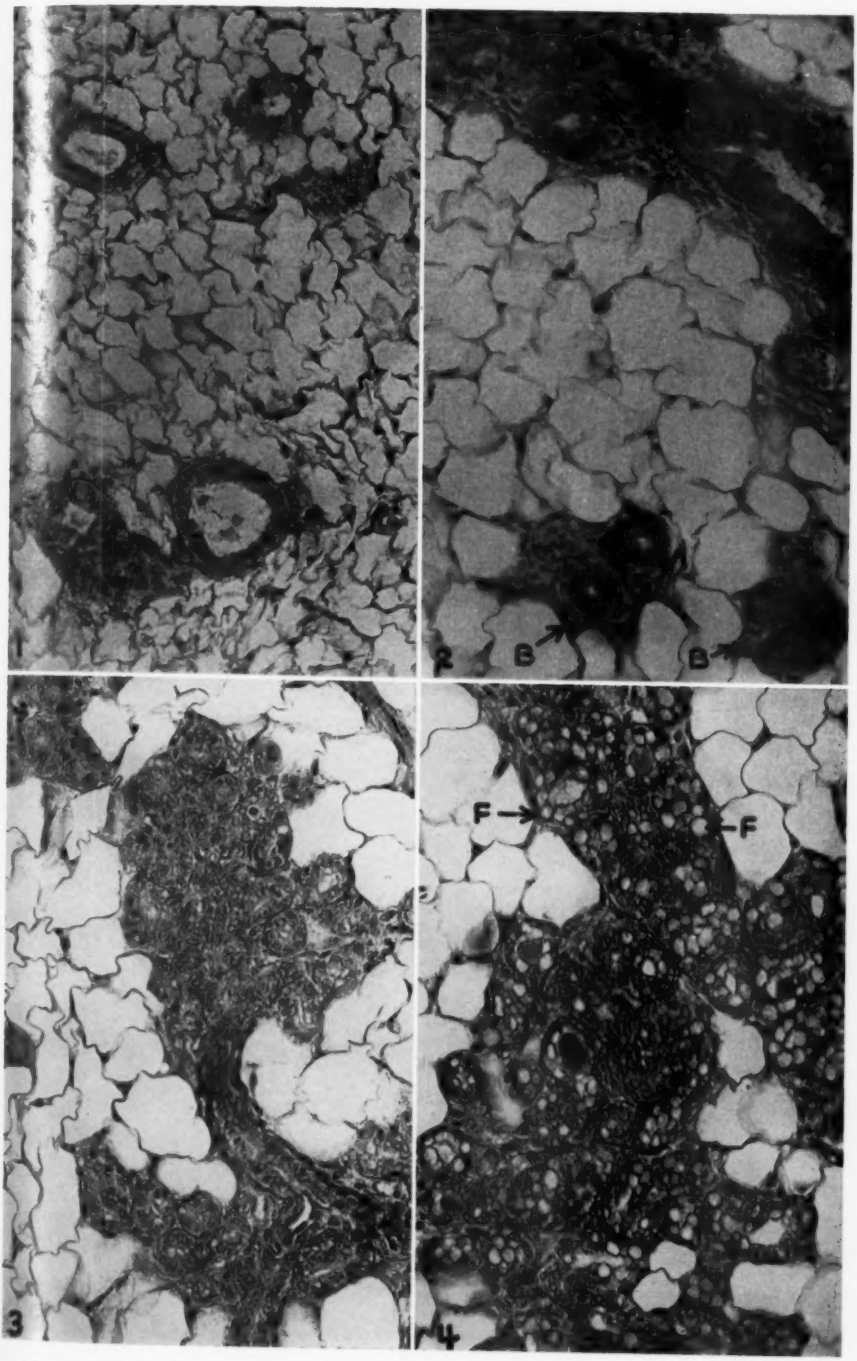

1
2
B
B
3
F
F
4

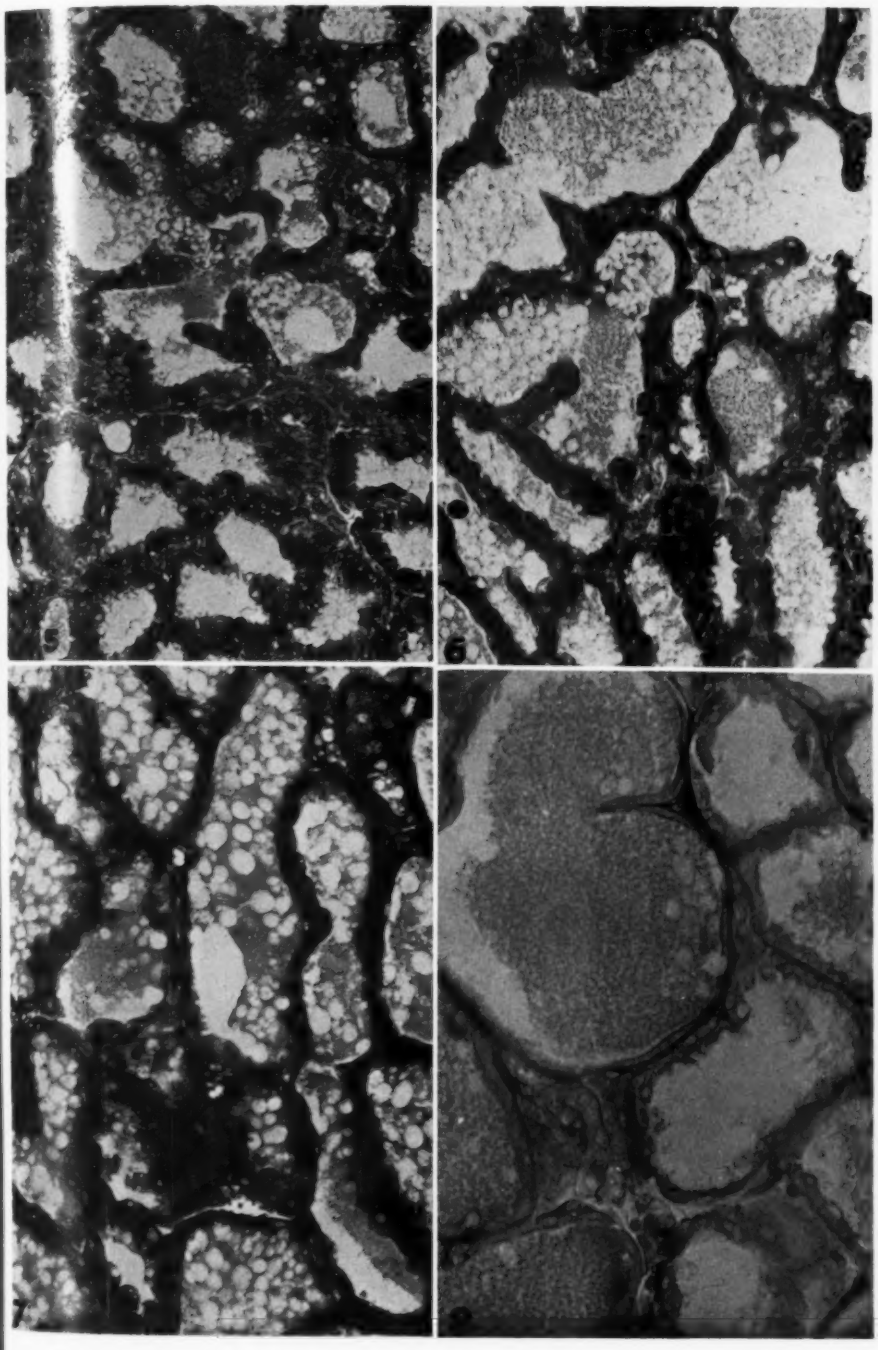
PLATE 42

FIGURE 5.—Mammary gland of mouse on 4th day of lactation. Alveolar lumens contain secreted material, including many fat droplets. Masson's stain. × 250

FIGURE 6.—Mammary gland of mouse on 15th day of lactation. Alveolar lumens contain granular secreted material and many fat droplets. Masson's stain. × 250

FIGURE 7.—Mammary gland of mouse on 15th day of lactation. Granular material in the lumens and most other tissue components give positive bromphenol blue stain. × 270

FIGURE 8.—Mammary gland of mouse on 15th day of lactation. Granular material in lumens and alveolar cells takes a moderately intense PAS stain; basement membranes take a strong PAS stain. × 270

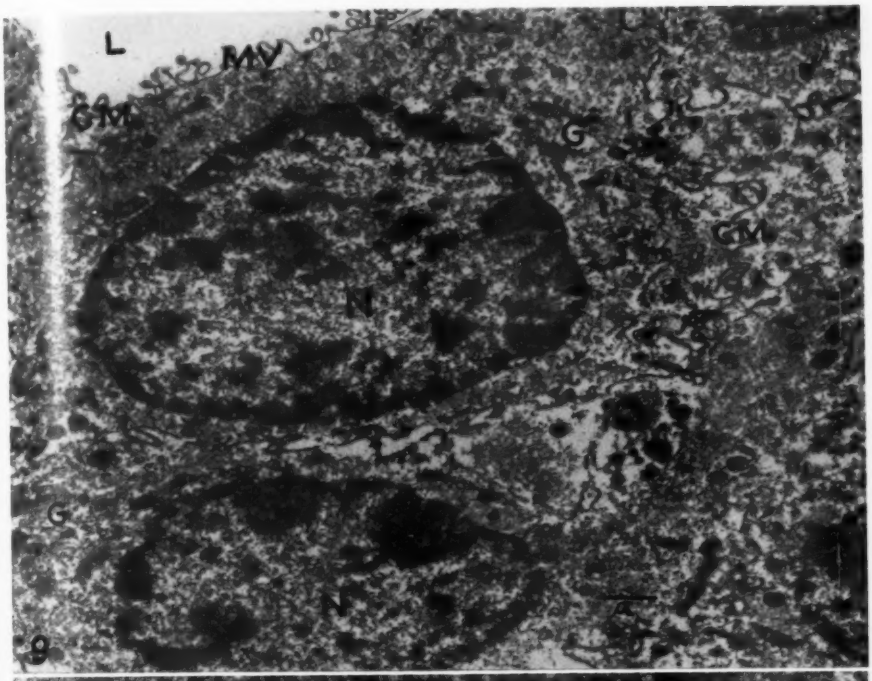


Figures 9 through 21 are electron micrographs

PLATE 43

FIGURE 9.—Epithelial lining cells of mammary duct of 3- to 4-month-old virgin C3H/Crgl mouse. Cell membranes (CM), nuclei (N), duct lumen (L), microvilli (MV), and Golgi apparatus (G). Endoplasmic reticulum and mitochondria sparse. Osmic acid fixed and stained with uranyl acetate. × 9,200

FIGURE 10.—Lumen of duct bud and parts of several epithelial cells from mammary gland of mouse pregnant 7 days. Lumen (L), nuclei (N), cell membranes (CM), mitochondria (M), and numerous ribonucleoprotein particles (R). Osmic acid fixed and stained with uranyl acetate. × 28,000


L
MV
GM
G
GM
N
G
N
9

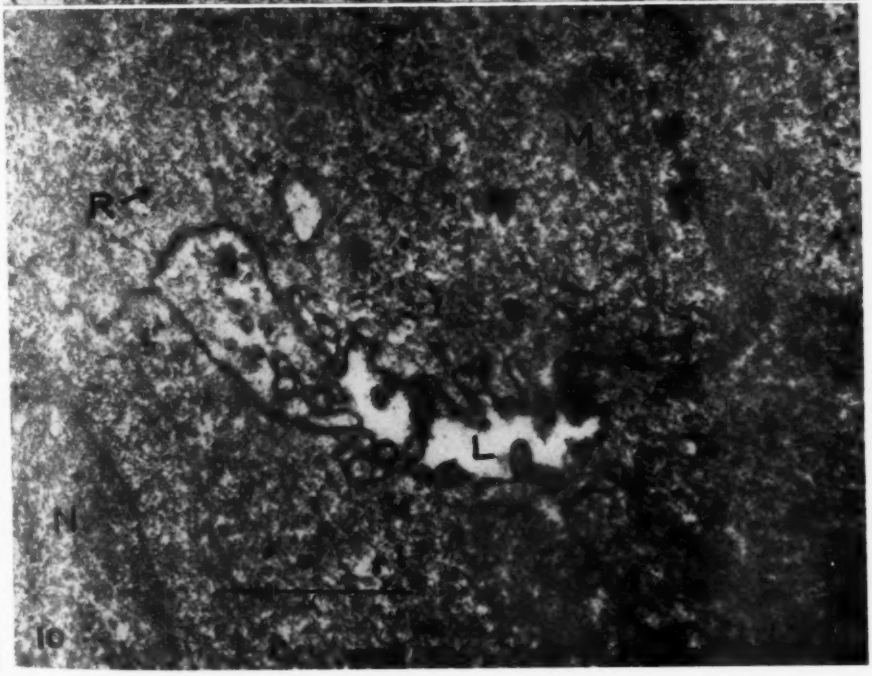

M
N
R
L
N
10

PLATE 44

FIGURE 11.—Epithelial cells lining a small alveolus from mammary gland of mouse on 14th day of pregnancy. Lumen (L) contains protein droplets, nuclei (N), nucleolus (NC), mitochondria (M), and endoplasmic reticulum (ER). "Pores" appear in nuclear membranes. Osmic acid fixed and stained with uranyl acetate. × 13,200

FIGURE 12.—Epithelial cells and alveolar lumen (L) of mouse on 14th day of pregnancy. Protein droplets (P), mitochondria (M), and ribonucleoprotein particles (R) are observed in the cytoplasm. Lumen contains protein droplets (P) and viruslike particles (V). Osmic acid fixed and stained with uranyl acetate. × 43,600

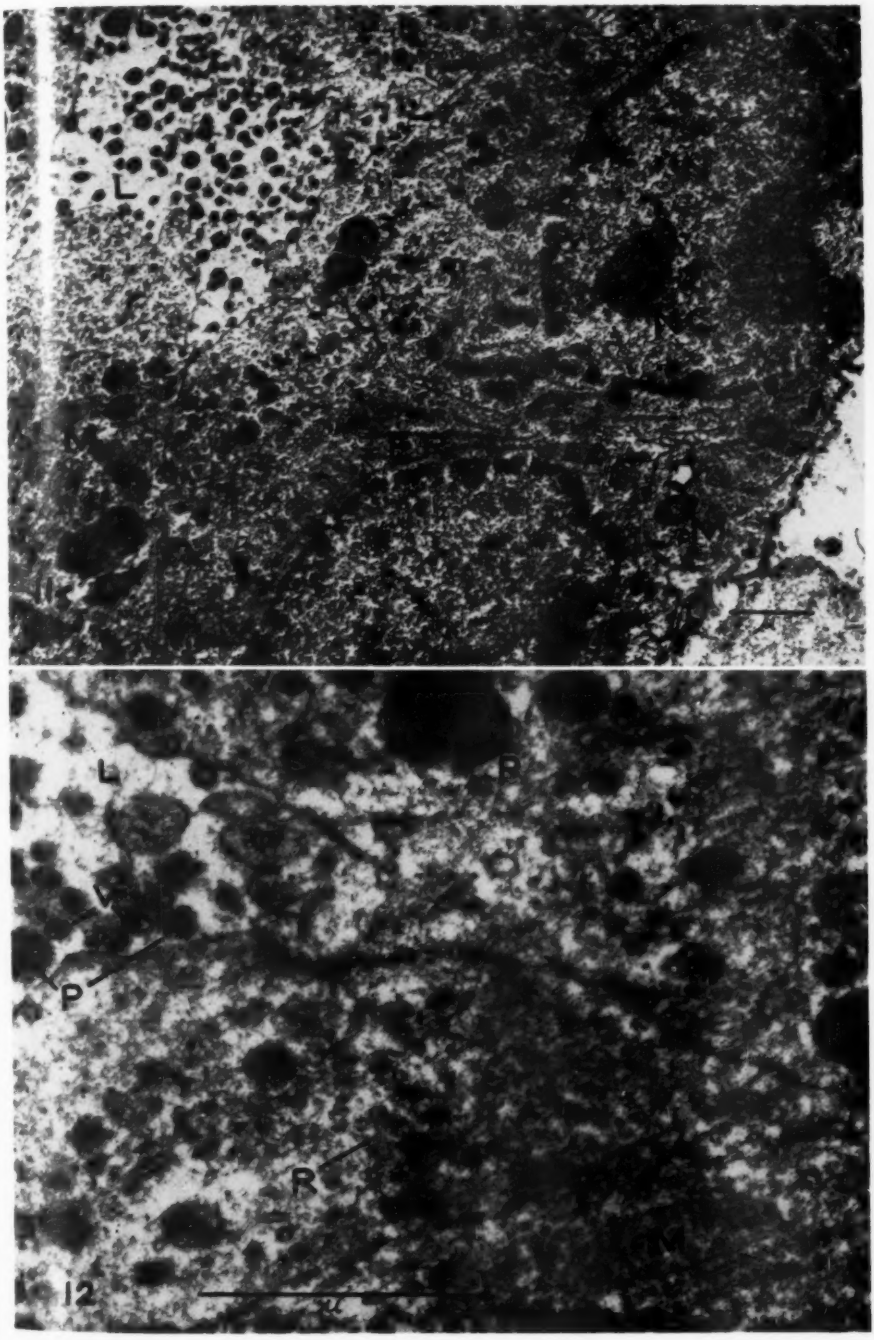

L
L
P
P
P
R
12

PLATE 45

FIGURE 13.—Epithelial cells lining alveolus of mammary gland of mouse on 19th day of pregnancy. Lumen (L) contains protein droplets. Nuclei (N) and fat droplets (F). Cell apices contain protein droplets (P). Osmic acid fixed and stained with uranyl acetate. × 13,200

FIGURE 14.—Part of an alveolar epithelial cell of mouse on 19th day of pregnancy. Fat droplets (F), protein droplets (P), mitochondria (M), and Golgi apparatus (G); small protein droplets associated with membranes of Golgi apparatus (*arrows*). Osmic acid fixed and stained with uranyl acetate. × 48,300

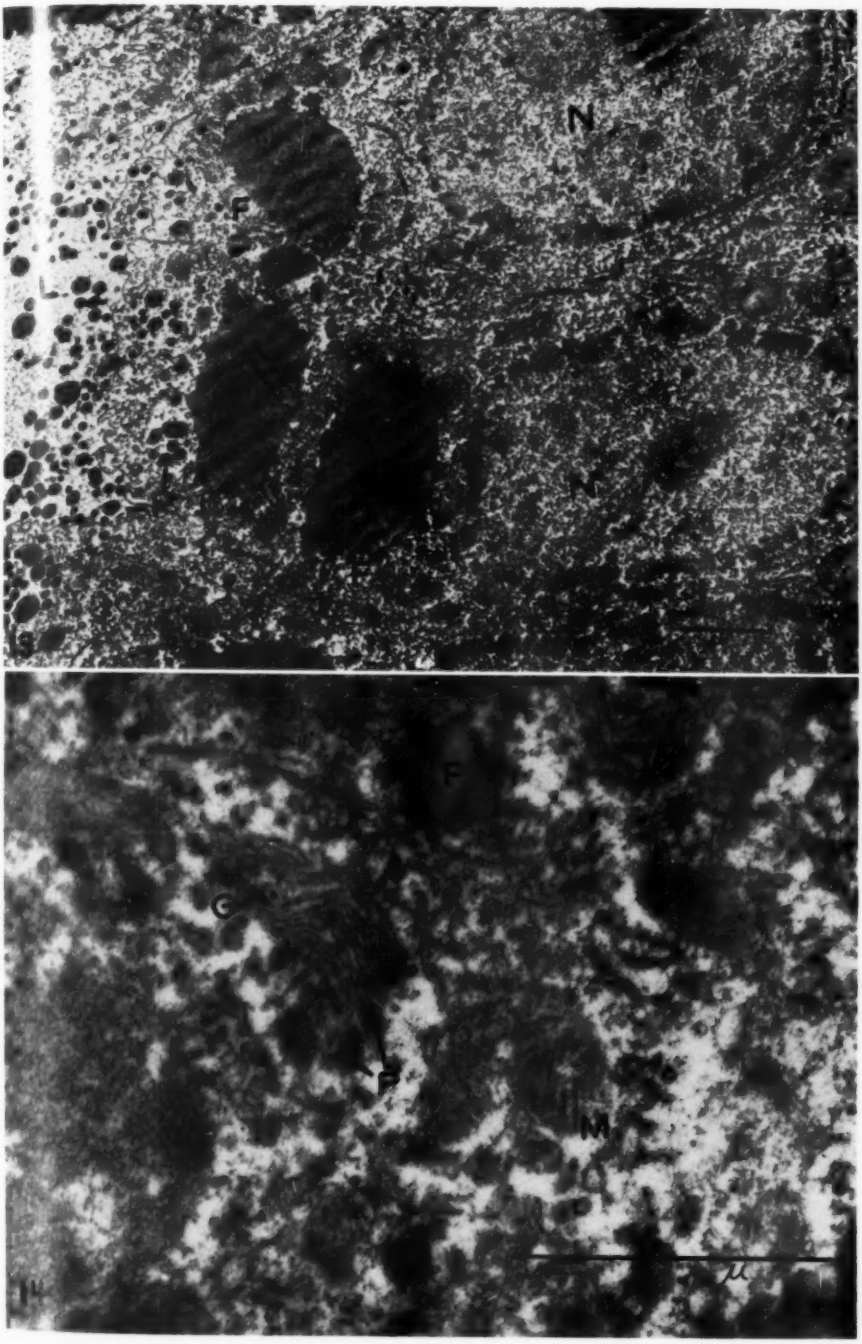

N
F
N
F
G
F
M
μ

PLATE 46

FIGURE 15.—Alveolar epithelial cell from the mammary gland of mouse on 4th day post partum. Lumen (L) contains protein droplets (P) and fat droplet (F); a membrane encloses part of fat droplet (*arrow*). Numerous mitochondria (M) and well-developed endoplasmic reticulum (ER) and Golgi apparatus (G). Osmic acid fixed and stained with uranyl acetate. × 12,000

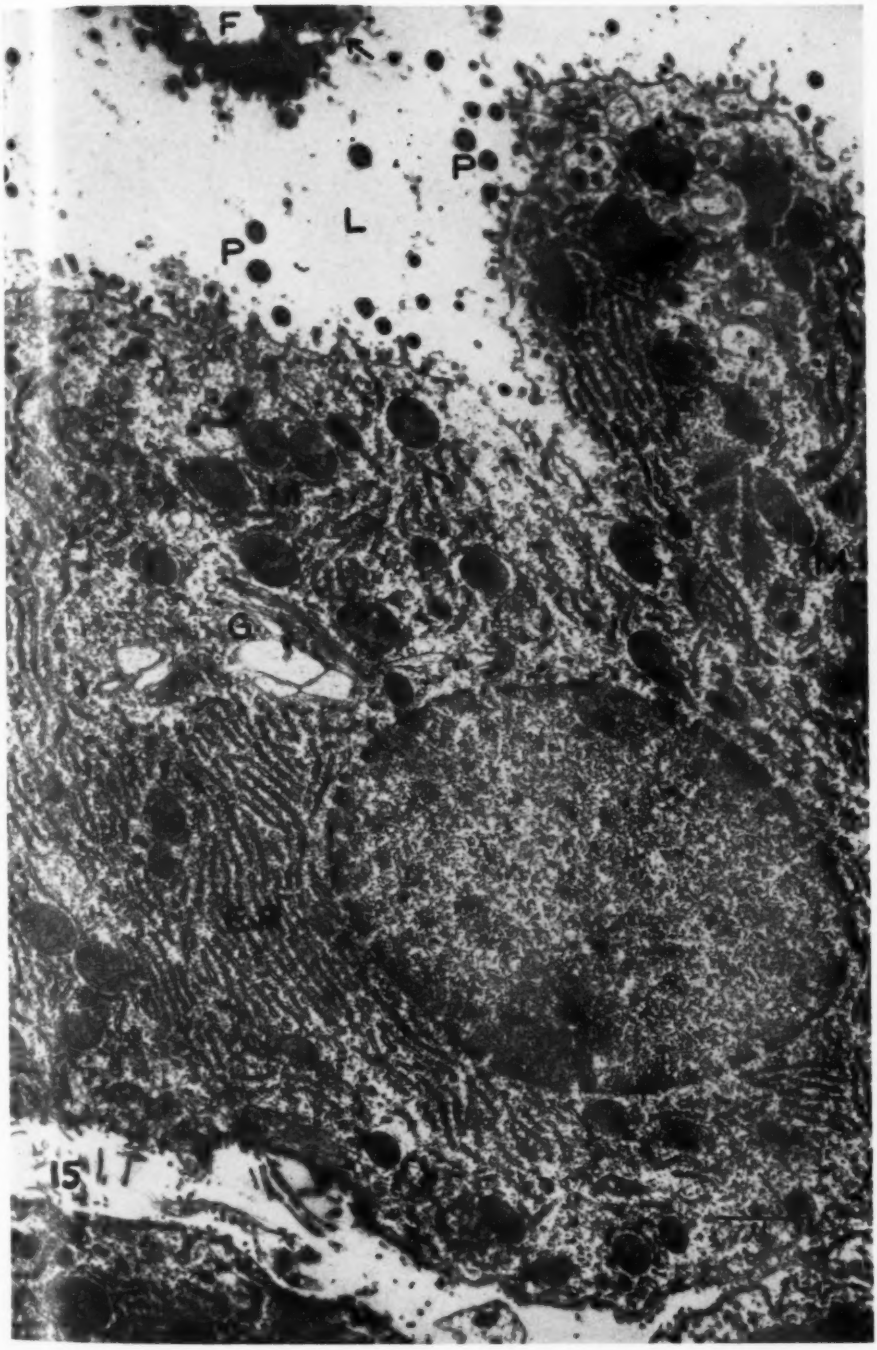

F
P
L
P
G
15

PLATE 47

FIGURE 16. Part of alveolar epithelial cell from the mammary gland of mouse on 4th day post partum. Lumen (L), mitochondrion (M), endoplasmic reticulum (ER), and Golgi apparatus (G) evident. Protein droplet (P) in the lumen; similar structures inside vacuoles of the cell apex and Golgi apparatus (*arrows*). Osmic acid fixed and stained with uranyl acetate. × 50,000

M
L
P
G
G
ER
G
μ

PLATE 48

FIGURE 17.—Alveolar epithelial cells of the mammary gland of mouse on 4th day post partum. Lumen contains protein droplets (P), and fat droplet (F) appears in process of being released from apex of cell. Osmic acid fixed and stained with uranyl acetate. × 13,000

FIGURE 18.—Part of apex of alveolar epithelial cell of mouse on 4th day post partum. Mitochondria (M), Golgi apparatus (G), nucleus (N), endoplasmic reticulum (ER), protein droplet (P), and viruslike particles (V). Osmic acid fixed and stained with uranyl acetate. × 30,800

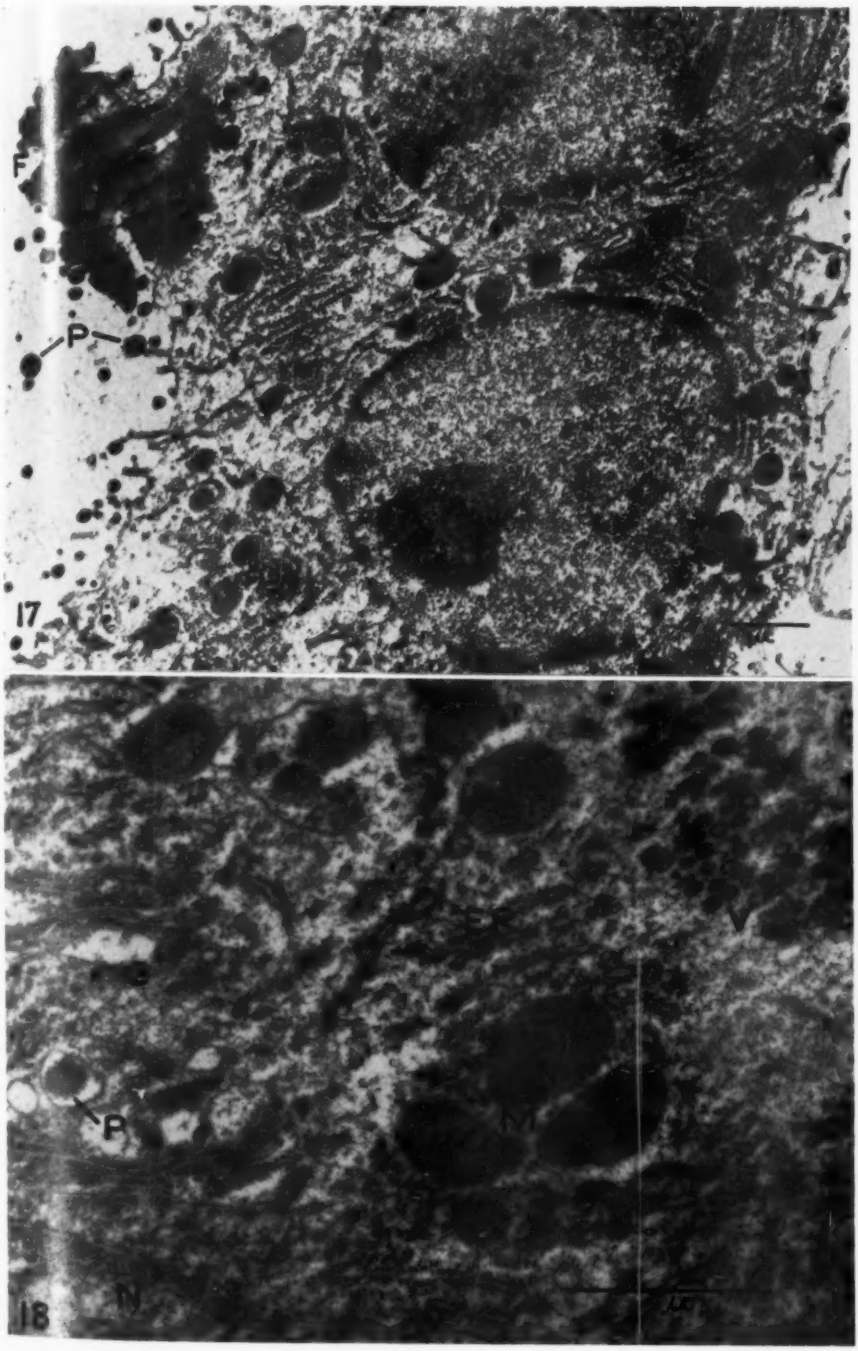

F
P
17
V
P
18
N

PLATE 49

FIGURE 19.—Alveolar epithelial cell of mouse on 15th day post partum. Protein particles (P) in the lumen and in cytoplasm (*arrows*). Nucleus (N), mitochondria (M), endoplasmic reticulum (ER), and Golgi apparatus (G). Osmic acid fixed and stained with uranyl acetate. × 16,600

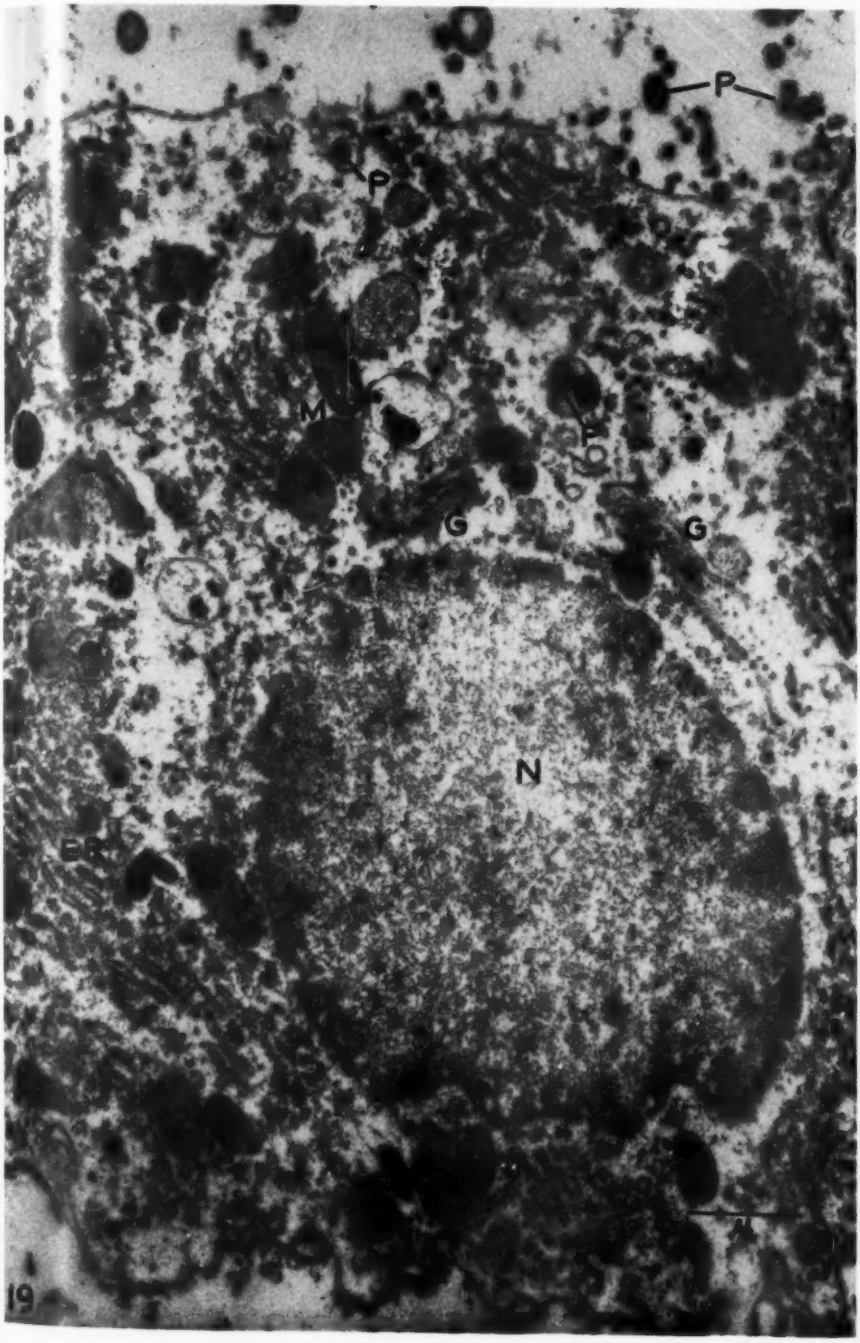
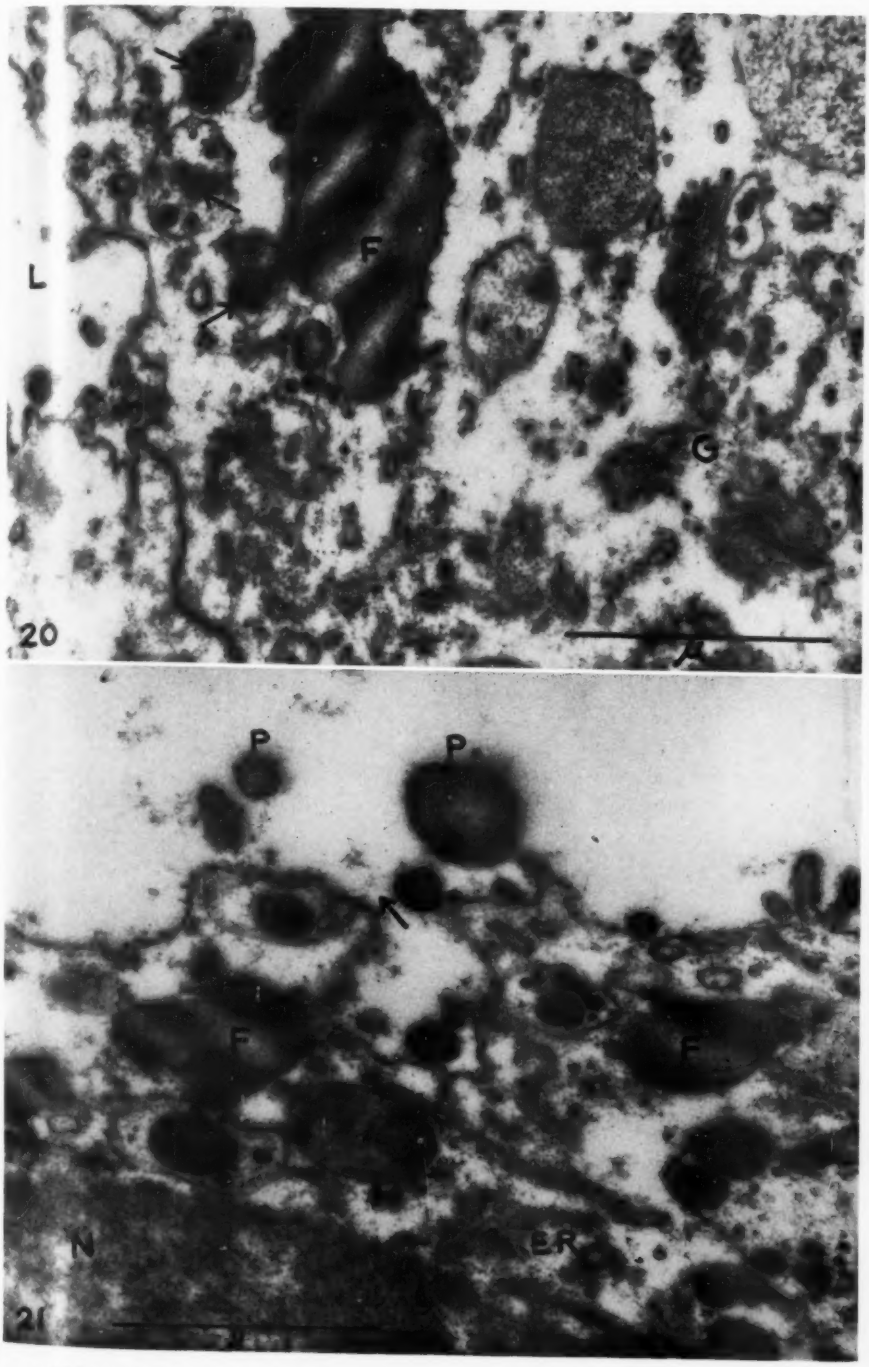

P
P
M
G
G
N
ER

PLATE 50

FIGURE 20.—Portion of apical cytoplasm of alveolar epithelial cell of mouse on 15th day post partum. Lumen (L), fat droplet (F), and Golgi apparatus (G). Protein droplets (*arrows*) are enclosed in vacuoles adjacent to cell membrane. Osmic acid fixed and stained with uranyl acetate. × 39,600

FIGURE 21.—Part of apical cytoplasm of an alveolar epithelial cell of mouse on 15th day post partum. Lumen contains protein droplets (P); similar droplets enclosed in vacuoles immediately adjacent to cell membrane. In center of figure (*arrow*), vacuole appears to be in process of releasing protein droplets into lumen. Fat droplets (F), mitochondrion (M), nucleus (N), and endoplasmic reticulum (ER). Osmic acid fixed and stained with uranyl acetate. × 39,600


L
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G
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P
P
F
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N
ER
21

Electron Microscopy of Milk Secretion in the Mammary Gland of the C3H/Crgl Mouse. II. Identification of Fat and Protein Particles in Milk and in Tissue [1, 2]

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SUMMARY

Air-dried mouse-milk films, frozen-dried mouse milk, frozen-dried mouse lactating mammary tissues, and osmium tetroxide-fixed lactating mammary tissues of the mouse were examined by electron microscopy. Air-dried and frozen-dried preparations treated with a solution of absolute methanol and ethyl ether (1:1) solubilized the fat droplets but failed to remove the protein particles. Tryptic digestion of formalin-fixed, methanol ether-treated grids of the same material removed the protein particles. The results established the proteinaceous and fatty characters of these 2 classes of milk particulates observed in the osmium tetroxide-fixed lactating mammary-gland tissue by electron microscopy.—J. Nat. Cancer Inst. 25: 423–437, 1960.

THE MILK within the alveoli of the lactating mammary gland of the mouse has strong histochemical reactions for protein, glycoprotein, and fat (*1*). Electron microscopic studies of the lactating mammary gland of the rat and mouse (*1–3*) had revealed two types of droplets or particles in the alveolar milk and the secretory cells: one type was large and homogeneous, its size and appearance suggesting a droplet of fat; the other was small and dense, appeared to form in the Golgi apparatus and was believed to be proteinaceous and to correspond to the casein particle seen by several workers in electron micrographs of centrifugally fractionated milk (*4–8*).

The proposed chemical identities of these two types of particles in electron micrographs of lactating mammary tissue were speculative, since direct proof of their chemical nature was lacking. The present study utilizes cytochemical methods and frozen-dried tissue to confirm the chemical identity of the fat and protein particles directly in electron microscopic images of thin-sectioned milk and mammary gland, and is a basis for further study of the endocrine control and cytomorphology of fat and protein synthesis in the mammary gland of the mouse.

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MATERIALS AND METHODS

In the present experiments the following materials were studied: (1) dried mouse-milk fractions separated by centrifugation; (2) frozen-dried whole mouse milk, embedded im methacrylate, and thin-sectioned; (3) frozen-dried mammary-gland tissue from lactating mice, embedded in methacrylate and thin-sectioned; (4) mammary-gland tissue from lactating mice, fixed in osmium tetroxide and prepared for electron microscopy in the usual way.

Dried mouse-milk fractions.—Mouse milk was obtained from lactating C3H/Crgl mice. Each donor mouse was anesthetized with sodium pentobarbital (*9*), and given a subcutaneous injection of 0.2 units of oxytocin (Pitocin: Parke, Davis & Company, Detroit, Mich.). After an interval of 3 to 5 minutes, drops of milk were expressed from the nipples, collected in microliter amounts in capillary tubes, and centrifuged at 7300 × g for 10 minutes to separate the light and heavy components. Samples were taken from the centrifugal and centripetal ends of the capillary tubes and stained for protein with bromphenol blue and for fat with Sudan IV. In addition, similar samples diluted 1:400 with distilled water were air-dried on carbon-coated copper specimen grids. Some of these grids were examined immediately in RCA EMU 2B electron microscope and others were treated with solutions of methanol-ether or trypsin [trypsin (2× crystalline), Nutritional Biochemicals Corporation, Cleveland, Ohio].

Fat droplets were solubilized and other lipide extracted by means of absolute methanol and ethyl ether (1:1) (*10*). Several grids were agitated for 5 minutes in this mixture, air-dried, and then observed in the electron microscope.

Proteolytic digestion was accomplished by a 1 percent trypsin solution (0.1 gm. of crystalline trypsin dissolved in 10 ml. of 0.1 M phosphate buffer, adjusted to *p*H 7.4). The partial solution of particles in the buffer was prevented by prior fixation in 10 percent formalin for 10 minutes. After fixation, the grids were incubated in the buffered trypsin solution for 1 to 12 hours at 38° C., washed briefly in distilled water, air-dried, and examined in the electron microscope. Control grids were incubated in the buffer alone.

As previously described, approximately 1 ml. of milk was collected from 12 lactating mice for chemical analysis of the heavy fraction. The milk was pooled in a small test tube and centrifuged at 7300 × g for 10 minutes. The upper layers were discarded, the heavy residue was resuspended repeatedly in 2 ml. amounts of distilled water, and then centrifuged until the final supernatant was clear. A small amount of this heavy, solid material was resuspended in 1 ml. of distilled water, pipetted onto carbon-coated specimen grids in 2 to 3 microliter amounts, air-dried, and examined in the electron microscope. The remainder of the washed, heavy fraction was then dried in a vacuum oven at 55° C. and analyzed in duplicate for carbon, hydrogen, nitrogen, sulfur, and phosphorus by

combustion methods.[5] The residue was subjected to spectrographic emission analysis.[6]

Frozen-dried whole mouse milk.—Small drops of undiluted milk from the nipples, collected into capillary tubes, were frozen-dried in open gelatin capsules, with a modified Glick-Malmström apparatus (*11*), and then embedded in *n*-butylmethacrylate *in vacuo*, according to a procedure to be described (*12*). For this purpose about 1 μl. of milk droplets was placed in open gelatin capsules, quenched in liquid nitrogen at −195° C., and then transferred to a drying chamber maintained at −70° C. in an alcohol dry-ice bath. The condenser of the drying chamber contained liquid nitrogen at a temperature of −195° C., and the distance between the surface of the milk and the condenser was about 2 cm. The milk was dried at a pressure of 10^{-5} mm. Hg for 6 hours, warmed to room temperature, and dried *in vacuo* for an additional 18 hours. After the drying was completed, the milk was infiltrated *in vacuo* with *n*-butylmethacrylate to which the catalyst, 2-4dichlorobenzoyl peroxide, was added. The liquid methacrylate was added directly to the drying chamber. Both the drying chamber and the methacrylate were first cooled to −70° C. to reduce the ability of the methacrylate to solubilize components of the specimen. The gelatin capsules containing the tissue blocks and liquid methacrylate were then removed from the drying chamber and the methacrylate was polymerized at 0° C. by an ultraviolet light. The blocks were sectioned with a Porter-Blum microtome set at 50 mμ and equipped with a plate-glass knife. Thin sections were floated onto a solution of 20 percent acetone in distilled water, and mounted on carbon-coated copper specimen grids. Some of the grids were then examined in the electron microscope, whereas others were subjected to methanol-ether and trypsin treatment. Grids were agitated in the methanol-ether mixture for 5 minutes, air-dried, and then examined in the electron microscope. Other methanol-ether-treated grids were fixed in 10 percent formalin for 10 minutes, and then incubated in the 1 percent trypsin solution for 1 to 12 hours before examination.

Frozen-dried mammary-gland tissue from lactating mice.—Lactating mammary-gland tissue was taken for biopsy from the mouse under sodium pentobarbital anesthesia (*9*) and the tissue was cut into 1 mm. cubes. The cubes were then frozen-dried according to the procedure described, embedded in *n*-butylmethacrylate *in vacuo*, thin-sectioned, floated onto carbon-coated copper specimen grids, and air-dried. Some grids were then examined in the electron microscope, but others before examination were given methanol-ether and trypsin treatment as previously described for thin-sectioned mouse milk.

Lactating mammary-gland tissue fixed in osmium tetroxide.—Other cubes of lactating mammary tissue, also obtained for biopsy, were fixed in 1

[5] Chemical analysis performed by Microchemical Laboratories, Department of Chemistry, University of California, Berkeley, Calif.

[6] The authors are indebted to Mr. Michael Hrenoff and to Professor L. A. Strait of the Spectrographic Laboratory, University of California Medical Center, San Francisco, for the spectrographic emission analysis.

percent osmium tetroxide buffered at *p*H 7.4 with acetate-veronal and with added sucrose, 0.045 gm. per ml. (*13*). These tissues were dehydrated in ethanol, embedded in *n*-butylmethacrylate, thin-sectioned, mounted on carbon-coated copper specimen grids, stained with uranyl acetate (*14*), and then examined in the electron microscope.

The entire series of experiments was repeated 4 times, with the exception of the chemical analysis of the heavy fraction which was done in duplicate.

RESULTS

Lactating Mammary-Gland Tissue Fixed in Osmium Tetroxide

Lactating mammary tissue (figs. 1 and 2), fixed in osmium tetroxide and prepared for electron microscopy in the customary way, revealed protein particles and fat droplets similar in appearance to those noted in previous studies (*1–3*). The protein particles, 30 to 400 mμ in diameter, were found in abundance in the milk within the alevolar lumens and in the cell apices. Some of the protein particles in the osmium-tetroxide-fixed tissue were homogeneously dense, whereas others displayed open, less dense centers (fig. 1). As had been observed in previous studies, the protein particles appeared to form in association with the Golgi apparatus (*1–3*). Most fat droplets were larger than the protein particles, varied typically in diameter between 500 and 8000 mμ, and were found in the alveolar milk and in the apices of the secretory cells.

Dried Mouse Milk Fractions

Whole mouse milk was separated into 3 fractions by high-speed centrifugation in capillary tubes. The lighter centripetal fraction was yellow-white, resembled butter, and stained red with Sudan IV, indicating a high concentration of fat. The heavier centrifugal fraction was grey-white and stained blue with bromphenol blue, indicating that it consisted largely of protein. The third fraction was clear and watery, and was located between the centrifugal and centripetal fractions.

Electron micrographs of air-dried films of the diluted heavy fraction of mouse milk revealed numerous small particles varying in diameter from about 30 to 400 mμ (fig. 3). Treatment of similarly dried films, with a mixture of absolute methanol and ethyl ether (1:1), had no effect on the particles except for a possible slight increase in electron density. Fixation of the methanol-ether-extracted grids in 10 percent formalin for 10 minutes, followed by incubation in a 1 percent trypsin solution for 12 hours, cleared the grids of the small particles. The particles were still present on control grids incubated for 12 hours in buffer alone. These findings indicated that the small particles in the dried films were protein. The results of elemental analysis by combustion methods of the washed heavy fraction are shown in table 1. The residue gave emission bands for

calcium and magnesium when subjected to spectrographic analysis. Electron microscopy of the material submitted for analysis revealed small particles identical to those previously seen in air-dried films of the heavy fraction of mouse milk.

TABLE 1.—Elemental analysis of the heavy particulate fraction of mouse milk by duplicate determinations—percent by weight

	I	II	Average
Carbon	44. 51	44. 60	44. 56
Hydrogen	6. 77	6. 78	6. 78
Nitrogen	12. 54	12. 62	12. 58
Sulfur	1. 28	1. 28	1. 33
Phosphorus	2. 00	2. 04	2. 02
Residue	10. 5	11. 30	10. 90
Total	77. 60	78. 72	78. 17

Electron micrographs of air-dried films of the diluted centripetal fraction of mouse milk revealed much larger droplets, variable in size and shape (fig. 4). A few small, dense particles, like those found in the centrifugal fraction, were also present and were identified as protein particles, trapped in the light fraction. Methanol-ether treatment of these grids removed the large droplets, but the smaller, dense contaminant particles remained. These results indicated that the large droplets were fat, whereas the small particles were protein.

Frozen-Dried Whole Mouse Milk

Electron microscopy of frozen-dried whole mouse milk collected from the nipples, embedded in methacrylate *in vacuo*, and thin-sectioned in the usual way again revealed two distinct kinds of particles (fig. 5), corresponding in size to those seen in the air-dried milk fractions. Large, ovate or round, low-density droplets were surrounded by many small, dense particles, suggesting identification with the fat and protein droplets or particles seen in osmium tetroxide-fixed tissue sections. The large droplets, but not the small particles, were removed by methanol-ether treatment (fig. 6). Furthermore, trypsin incubation of formalin-fixed, methanol-ether-treated grids for 12 hours removed the remaining small particles. Partial digestion of the small particles was accomplished after 2 hours of incubation in the trypsin solution (fig. 7); only a small amount of granular debris remained after 12 hours of incubation. Control incubation in buffer alone had no effect on the small particles. These results indicate again that the small droplets are protein and the large ones are fat.

Frozen-Dried Mammary Tissue From Lactating Mice

Unfixed frozen-dried lactating mammary tissue, methacrylate-embedded and thin-sectioned, was examined by electron microscopy in the untreated state, as well as after treatment with methanol-ether and trypsin (figs.

8 to 10). The results were similar to those obtained from frozen-dried whole mouse milk. The protein and fat droplets were visible in the alveolar lumens adjacent to the apical margins of the secretory cells (fig. 8). Methanol-ether treatment removed the fat droplets, but the protein particles remained (fig. 9). Subsequent incubation in the trypsin solution again removed the protein particles (fig. 10), whereas incubation in the buffer alone had no effect. Tryptic digestion was essentially complete in 12 hours, leaving only a small amount of granular debris. These results demonstrate that the large droplets and small particles in the alveolar milk are fat and protein respectively.

DISCUSSION

In the present experiments, methanol-ether and trypsin treatment of air-dried milk fractions, frozen-dried whole mouse milk, and frozen-dried lactating mammary tissue of the mouse was carried out to confirm the chemical identification of the protein particles and fat droplets observed in osmium tetroxide-fixed mammary tissue. Methanol-ether and trypsin were applied directly to the preparations on the carbon-coated specimen grids, and the results observed with the electron microscope.

Frozen-dried milk and lactating mammary tissues were used in these procedures because the chemical methods were unsuccessful on materials fixed in osmium tetroxide and dehydrated in alcohol. Direct embedding in methacrylate *in vacuo* prevented contact of the tissue with moist room air, which could result in additional chemical and physical artifacts.

The protein particles were not solubilized and were essentially unaltered after the methanol-ether treatment of air-dried milk films, of frozen-dried whole milk, and of frozen-dried lactating mammary tissue, indicating that these particles contained little if any lipide. Under the same circumstances the fat droplets were removed. Subsequent tryptic digestion of the methanol-ether-treated grids removed the protein particles, indicating that they were composed mainly of protein. The diameters of the fat particles and of the protein particles were similar in air-dried milk films, frozen-dried milk, frozen-dried tissue, and osmium tetroxide-fixed lactating mammary tissue, indicating the identity of the respective classes of particles in the several different preparations.

The present results confirm previous assumptions that in osmium tetroxide-fixed lactating mammary tissue (*1–3*), the class of small particles is protein and the class of larger particles is fat.

Chemical analysis of the washed heavy fraction of mouse milk gave a nitrogen value of 14.1 percent after correction for residue; $\frac{(12:58)}{100-10.9}(100) = 14.1$ percent (*see* table 1). This finding is consistent with the interpretation that the small heavy particles are composed largely of protein.

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PLATE 51

FIGURE 1.—Electron micrograph of lactating mammary-gland epithelial cells of mouse. Protein particles (P) and fat droplets (F) in the lumen *left* and in apical cytoplasm. Cell membrane (CM), ergastoplasm (ER), mitochondria (M), and nucleus (N) are indicated. Osmic acid-fixed and stained with uranyl acetate. × 13,000

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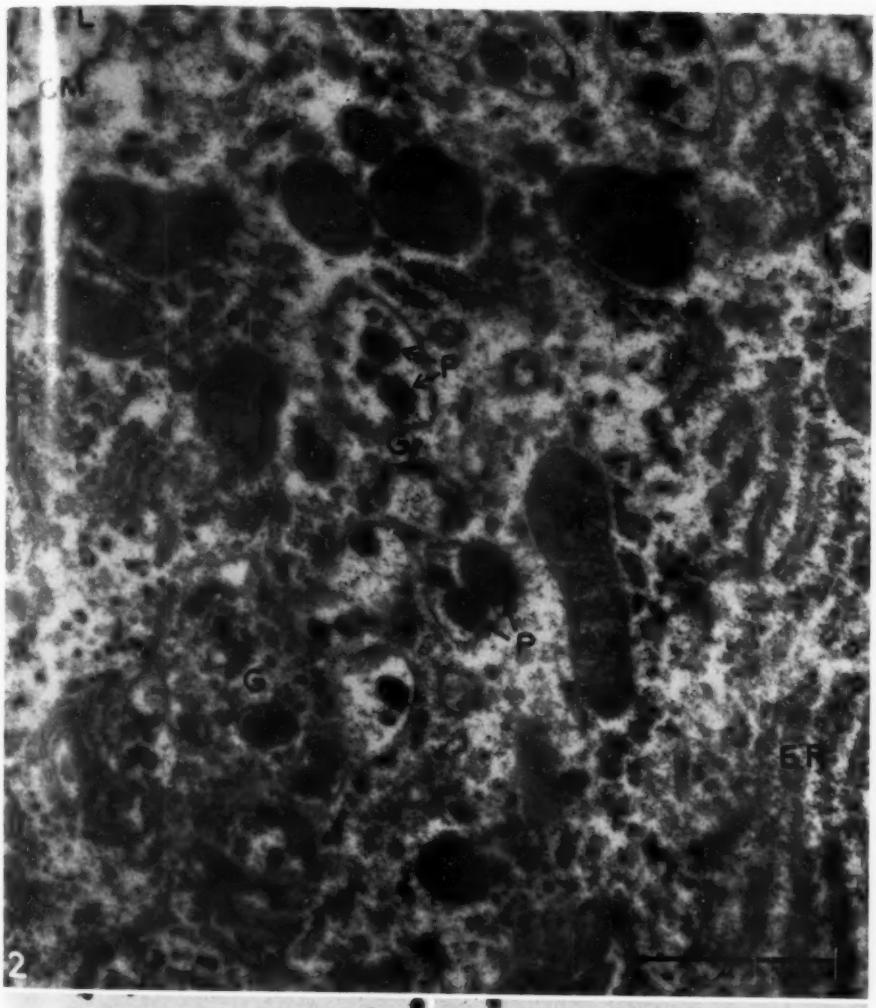
PLATE 52

FIGURE 2.—Electron micrograph of part of lactating epithelial cell from mouse mammary gland. *Lower left* shows a large portion of Golgi apparatus (G). Protein particles (P) are inside of Golgi vesicles. Lumen (L), cell membrane (CM), mitochondria (M), and ergastoplasm (ER) indicated. Osmic acid-fixed and stained with uranyl acetate. × 31,000

FIGURE 3.—Electron micrograph of air-dried film of diluted heavy protein fraction of mouse milk. Numerous spherical protein droplets visible. × 13,000

FIGURE 4.—Electron micrograph of air-dried film of diluted light fat fraction of mouse milk. Fat droplet (F) and a few protein particles (P) indicated. × 13,000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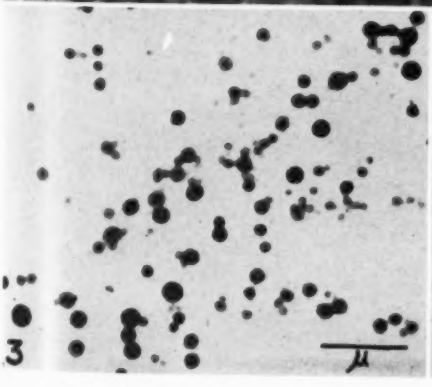
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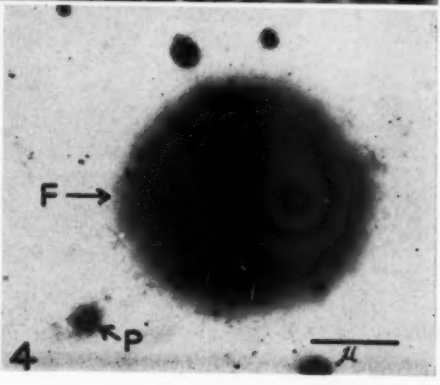
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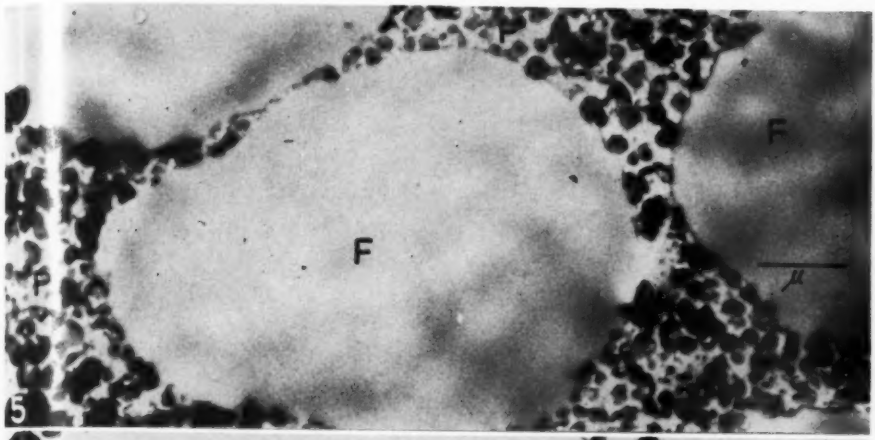
PLATE 53

FIGURE 5.—Electron micrograph of frozen-dried, methacrylate-embedded, thin-sectioned mouse milk. Fat droplets (F) and protein particles (P) indicated. *Note* fat droplets are considerably denser than relatively less dense methacrylate-filled spaces between protein particles. × 13,000

FIGURE 6.—Electron micrograph of preparation similar to figure 5 after treatment with methanol-ether. *Note* fat droplets (F) have been removed; protein particles (P) still intact. × 13,000

FIGURE 7.—Electron micrograph of preparation similar to figure 6 after only 2 hours of incubation in a 1 percent trypsin solution. Digestion of protein droplets (P) nearly complete. After 12 hours in trypsin, only small amount of granular debris remained. Location of fat droplets, previously removed with methanol-ether, is indicated (F). × 13,000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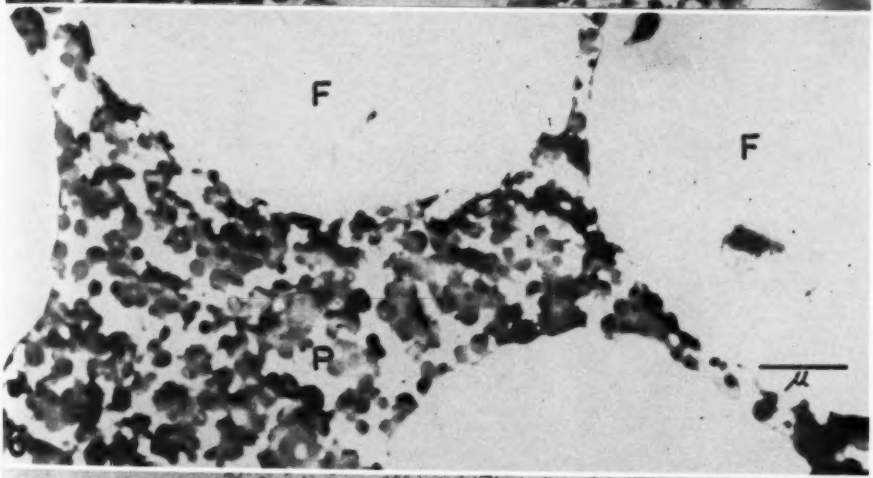
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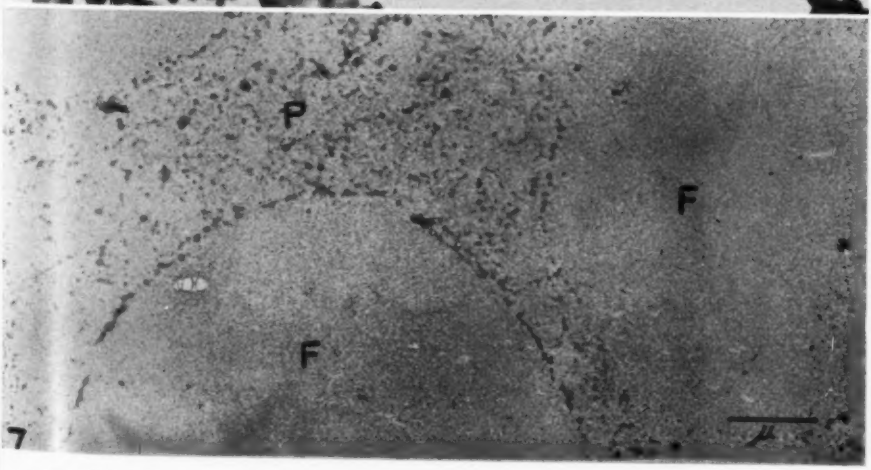
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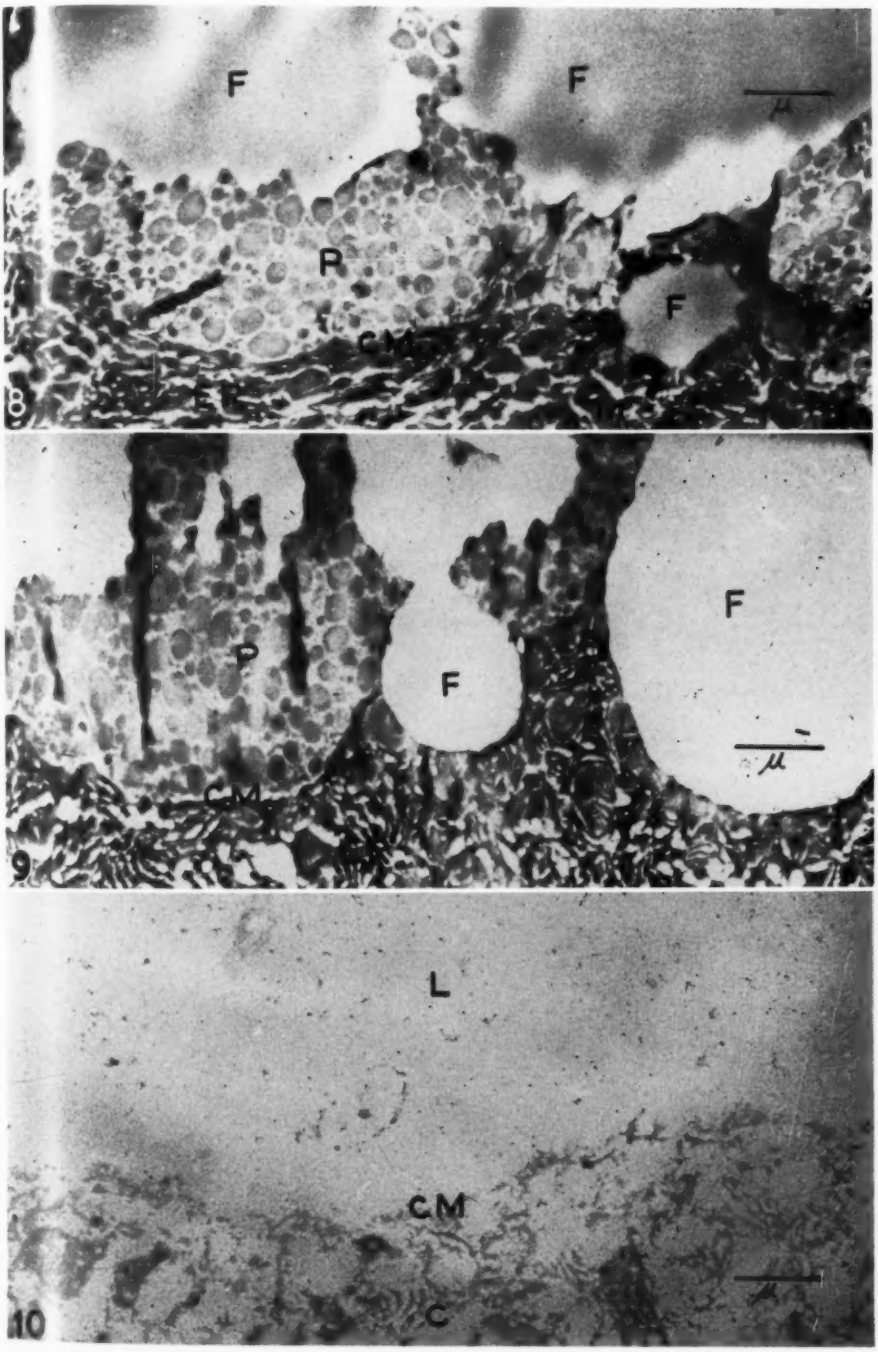
PLATE 54

FIGURE 8.—Electron micrograph of frozen-dried, methacrylate-embedded, thin-sectioned, lactating mammary-gland tissue of mouse. In this and succeeding figures apical cytoplasm is visible in the *lower* one third of figure; alveolar lumen visible in *upper* two thirds. Fat droplets (F) and protein particles (P) shown in the alveolar lumen. *Note* relatively empty, methacrylate-containing spaces within cell and between protein droplets are considerably less dense than fat droplet. Ergastoplasmic sacs (ER), mitochondria (M), and cell membranes (CM) also shown. × 13,000

FIGURE 9.—Electron micrograph of frozen-dried preparation similar to figure 8 after treatment with methanol-ether. *Note* that the fat droplets (F) have been removed; protein particles (P) are still intact. Also shown are the cell membrane (CM) and ergastoplasm (ER). × 13,000

FIGURE 10.—Electron micrograph of frozen-dried preparation similar to figure 9 after 12 hours' incubation in 1 percent trypsin solution. Digestion of the protein particles is complete, with only small amount of granular debris remaining in lumen (L). Cell membrane (CM) and partially digested cytoplasm (C) are indicated. × 13,000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Announcement

Manuscripts Approved for JNCI, June 15 to July 15, 1960

"Lung tumors produced by intratracheal inoculation of polyoma virus in Syrian hamsters," by Alan S. Rabson, William J. Branigan, and Frances Y. Legallais.

"Note on results of Linder and Klein with coisogenic resistant lines of mice," by George D. Snell.

"Mechanism of experimental tumorigenesis. VI. Ultrastructural alterations in mouse epidermis caused by locally applied carcinogen and dipole-type tumor promoter," by Kai Setälä, Lauri Merenmies, Mauri Nyholm, and Leo Stjernvall.

"Exchange of radioactive magnesium in Walker carcinoma: A note," by Terence A. Rogers, Frances L. Haven, and Parker E. Mahan.

"Effects of cortisone in tumor transplantation," by George E. Moore, Tatsuhei Kondo, and Robert J. Oliveer.

"Continuously propagating cells derived from normal mouse bone marrow," by Daniel Billen and G. A. Debrunner.

"Some factors involved in the induction or growth of testicular tumors in BALB/c mice," by H. B. Andervont, M. B. Shimkin, and H. Y. Canter.